The Elongator Complex Negatively Regulates Polarized Secretion

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Intracellular transport is fundamental for eukaryotic life and the function of regulators of transport is evolutionarily conserved. Rab proteins are a family of key regulators of vesicle and organelle transport, which aid in cell proliferation and cell survival. Functional conservation has allowed the simple single celled eukaryote, *Saccharomyces cerevisiae* to serve as a model system to study fundamental features of eukaryotes, including intracellular transport. An area of interest in the Collins laboratory is studying the signals that regulate membrane transport. The work presented in this thesis describes the negative regulation of exocytosis by the Elongator complex and the urmylation conjugation system. Deletion of the components of either system suppresses the temperature sensitivity of mutants defective in exocytosis. The Elongator complex has two proposed catalytic activities: acetyltransferase activity and SAM (*S*-adenosylmethionine) radical activity. We have found this complex is a cytoplasmic resident and therefore we propose that the relevant targets of these activities are in the cytoplasm or on cytoplasmic organelles that may influence membrane transport. Genetic analysis indicates that the profile of suppression of exocytic mutants caused by loss of Elongator complex function is similar to the suppression caused by overexpression of the exocytic Rab GTPase *SEC4* and the t-SNARE *SEC9*. This suggests that the physiologic output of Elongator

complex function may influence Sec4p function or SNARE function. These two functions are involved in the regulation of vesicle targeting and fusion, which may place Elongator function at this stage of the regulation of membrane transport. Other studies have reported that Elongator function also influences transcription and translation events. Our current model suggests that Elongator may serve as a signaling complex to regulate exocytosis in response to the translational capacity of the cell.

BIOGRAPHICAL SKETCH

The author was born in New Haven, CT on February 11, 1980 to the parents of Dale and Scott Rahl. He was raised in Mystic, CT, along with an older brother of two years, Matthew, and a fraternal twin brother, Daniel. His education started at S.B. Butler Elementary School and Carl C. Cutler Middle School. From there, he went to Robert E. Fitch High School in Groton, CT. During his time at Fitch High School he realized his passion for science. The Advanced Placement Biology, Mrs. Charmaine Mizak, really opened his eyes to the type of questions and phenomena that can be studied in the laboratory setting. She has close ties with researchers at the Pfizer Inc Central Research in Groton, CT, and was able to expose her students to the exciting world of scientific discovery as opposed to the usual high school science classes focusing solely on memorizing scientific theory.

Spring and summers were absorbed by baseball, either traveling to Fenway Park in Boston or Yankee Stadium in the Bronx to watch games, or playing in games throughout southeastern Connecticut. The times he wasn't near a baseball diamond, he loved to hang out with friends at the beach and swimming in the ocean. He played soccer in fall and basketball or pond hockey in winter. The countless hours of playing sports instilled a deep sense of teamwork and taught him how teamwork is a powerful way to accomplish difficult tasks.

The author enrolled at Merrimack College in North Andover, MA upon graduating from high school. His four years at Merrimack was an exciting time as he developed a deep friendship with a number of individuals that will last his lifetime. He majored in biochemistry and began learning about the methods and technology that could be used to understand different aspects of biology. Semester breaks were spent doing research at Pfizer Inc in Groton, CT. His first laboratory experience was in the

Salt Selection and Crystallization group under the mentorship of Dr. Glenn Williams. The remaining three summers were spent in the Biopharmaceutics group under the mentorship of Dr. Steven Sutton and Ms. Jennifer McCarthy. During his senior year at Merrimack College, the author studied mitochondrial biology in the laboratory of Dr. Josephine Modica-Napolitano.

After completion of the requirements to receive a bachelor's of science degree from Merrimack College, he accepted an offer to pursue a Ph.D. degree in the field of Pharmacology at Cornell University in Ithaca, NY. He joined the laboratory of Dr. Ruth Collins in the Department of Molecular Medicine and received excellent mentorship to develop into an independent scientist. He studied the regulation of intracellular transport and developed an understanding for using genetics, cell biology, molecular biology and biochemistry as tools to approach a scientific question. Upon completion of his degree requirements at Cornell, the author will get additional training at the Whitehead Institute at the Massachusetts Institute of Technology, Boston, MA in the laboratory of Dr. Richard Young. He will receive training in the field of genomics. He is excited to move to Boston, as this will bring him closer to his brothers and parents.

Dedicated to Dale and Scott Rahl, my caring parents who have taught me so much and shaped me into the person I am today. I love you.

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I would like to thank my thesis advisor, Ruth Collins. Ruth was a great mentor and taught me so much during my time in her lab. I joined her lab with little knowledge of molecular biology, cell biology and genetics techniques and through her mentorship I developed a very good foundation in these areas of research. I cannot thank her enough for her guidance in teaching me how to approach a scientific problem. I feel like I have really grown as a scientist during my time in her laboratory and she has provided me with an excellent scientific foundation for my future opportunities.

I am greatful for having an excellent thesis committee. Dr. Richard Cerione, Dr. Anthony Bretscher and Dr. Geoffrey Sharp provided me great advice for my project as well as giving me excellent career advice.

I would like to thank the past and current members of the Collins laboratory for all of their help, support, and discussions: Monica Calero, Catherine Chen, Carole DeRegis, Ian Berke, Chris Heger, David Cragun, Chantal Binda, Wenyan Zhu, Mengqiao Wang, and Eric Williams. Monical Calero was a senior graduate student when I joined the lab and she taught me a lot of the techniques used in the laboratory. Ian Berke is a postdoctoral researcher in the lab with excellent knowledge of working with proteins. He has taught me so much during his time in the lab, and I would like to thank him for that. He also generated the Hos3p-GFP and his_6-FLAG-Urm1p constructs that I used in my studies. Chris Heger is a graduate student that has been a member of the lab for my entire time in graduate school. Catherine Chen and Carole DeRegis are also graduate students in the laboratory for the majority of my time here. Chris, Catherine and Carole all taught me so much in terms of techniques and experimental ideas. A very nice aspect of the lab is that each person generally has a particular affinity for certain techniques or areas of research, and Chris, Catherine and

Carole taught me a lot from their respective strengths. I worked with Mengqiao Wang and Eric Williams while they were rotation students in the laboratory and I used constructs that they generated for my studies. I would like to thank Uya Chalaanbaatar who was an undergraduate that worked with me for two years. She was a very talented undergraduate who generated strains and constructs that were useful to my studies. She was a good friend and I wish her the best during her graduate work. Chantal Binda and Wenyan Zhu were technicians in the laboratory and they were extremely helpful during my time in the lab. The Collins lab is a great atmosphere for research and everybody in the lab has been a great friend.

There were so many people outside of my committee members and the Collins lab that helped me during my graduate studies that I would like to thank. Dr. Gary Whittaker and Benjamin Briggs taught me so much about microscopy, which has proven to be a very important part of my studies. I would like to thank Dr. Holger Sondermann, a faculty member of the Department of Molecular Medicine, who has offered excellent advice during my graduate work. Dr. David Pruyne, a research associate in the Bretscher laboratory, taught me so much during my time in the lab and has been an extremely useful resource during my time in the lab. During my graduate work Dave taught me the basics of immunofluorecence microscopy in yeast and has been extremely helpful for technical discussions for method development. I would like to thank members of the Whittaker lab. We interact frequently with the Whittaker lab and they have been helpful during my studies. Dr. Patrick Brennwald, University of North Carolina at Chapel Hill, who provided me with a number of plasmids that I used during my studies in Chapter 5. These plasmids helped a great deal in my genetic analysis of the Elongator complex. I would like to thank Carol Bayles who trained me on the confocal microscope in Biotechnology Resource Center at Cornell and

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I would like to thank the American Heart Association for providing funding for my final year of graduate work (0615699T). Also, the National Institutes of Health for providing funding from the Molecular Medicine training grant. In addition, the faculty members and staff of the Department of Molecular Medicine at Cornell for provided excellent help and support during my time in the department.

Lastly I would like to thank my friends and family who have given me amazing support during my graduate studies. My girlfriend, Jacqueline Fremont, has been amazing during my graduate work. She has understood the long work hours and the emotional ups and downs of research life and has been extremely forgiving. Lastly, I would like to thank my parents and brothers. They have been the best during my time here and were always available to talk to me to give me a pep talk. I could not have done it without their continued support.

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Chapter 1. Introduction

Regulation of intermembrane transport is an evolutionarily conserved eukaryotic feature

A hallmark of eukaryotic cells is their ability to organize their contents in membrane bound organelles. Cellular building blocks, including proteins, lipids, and nucleic acids, flow between compartments of the intermembrane system. This allows for the uptake of macromolecules from the extracellular space via the endocytic system and for the transport of synthesized molecules out of the cell in a series of regulated steps through the secretory pathway. Vesicles facilitate both of these processes.

Membrane enclosed vesicles serve as a major cargo carrier between different organelles. Vesicles bud from a donor compartment and travel to an acceptor compartment where the contents are deposited (Figure 1.1) [1]. Biosynthetic flow originates in the endoplasmic reticulum (ER) where vesicles transport molecules to the Golgi apparatus (Figure 1.2). Once in the Golgi, molecules are sorted and can be trafficked to the cell surface in secretory vesicles or transported to the endosomal system and the late endosomes.

Molecules that are targeted for late endosomes are generally destined for degradation and subsequently transported to the lysosome or vacuole, depending on cell type. Molecules can be internalized through the endocytic pathway where early endosomes carry cargo towards the cell interior. From here, the molecules enter the sorting endosomes and are either trafficked to the Golgi for transport in the secretory pathway or targeted for degradation in the lysosome.

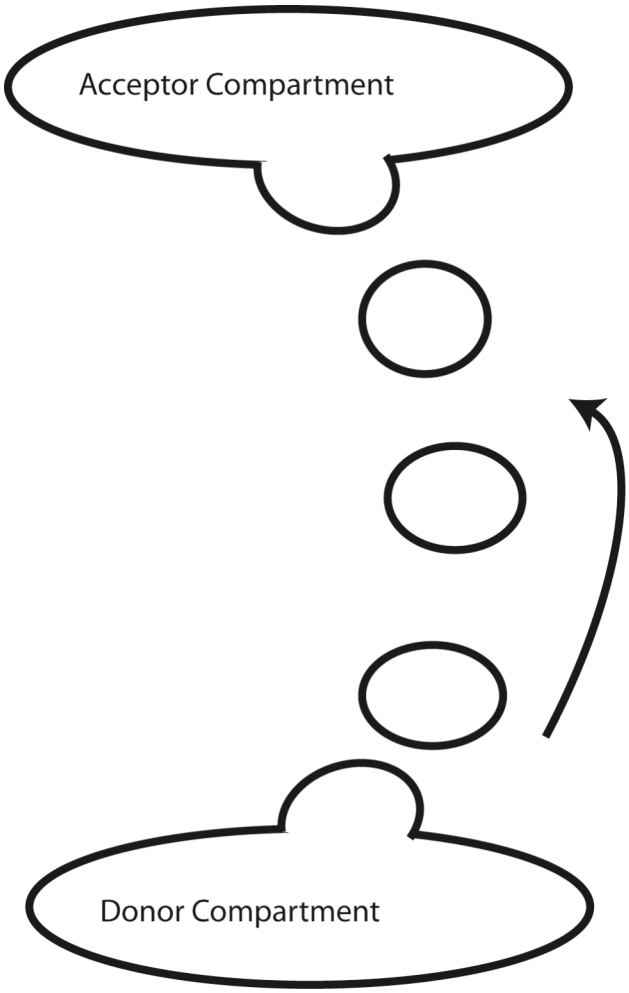

Figure 1.1. Intracellular transport between compartments. Vesicles bud from the donor compartment and transport cargo to the acceptor compartment, where vesicles fuse and contents are released.

The regulation of transport between organelles is crucial for maintaining cellular homeostasis. Due to the importance of this function, many of the proteins that regulate these steps are evolutionarily conserved from the single celled eukaryote *Saccharomyces cerevisiae* (yeast) to mammals, as outlined in Figure 1.3. Shown are

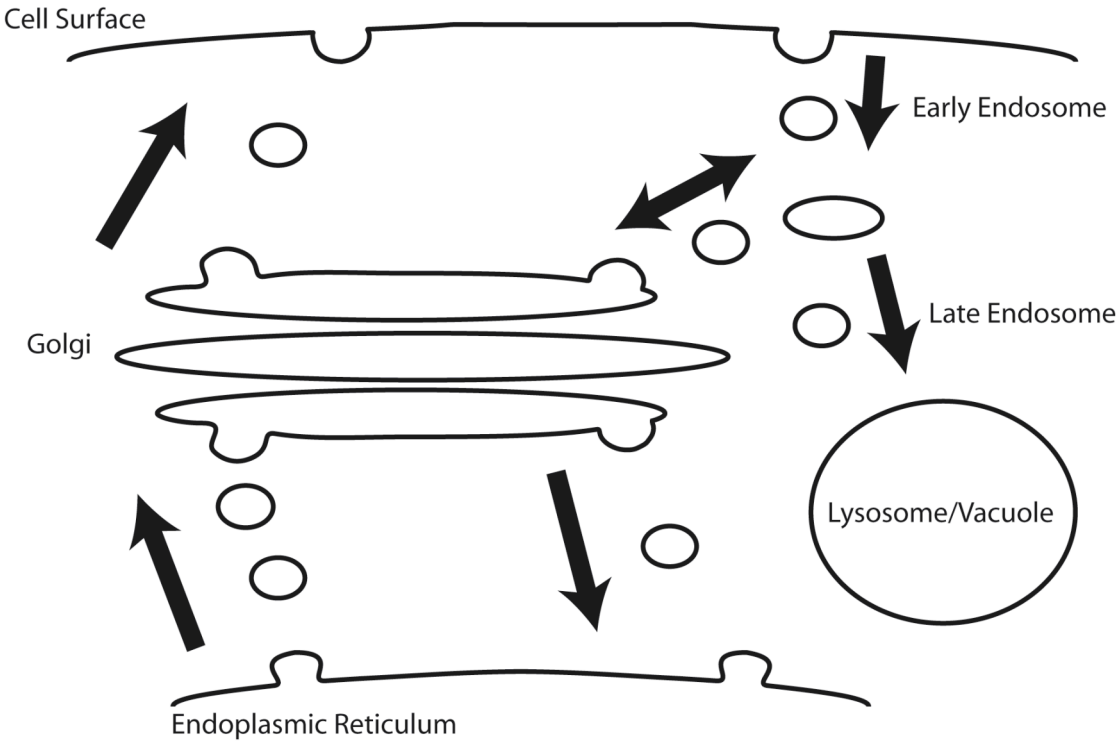

Figure 1.2. Macromolecule transport stages through the intermembrane system. The transport of molecules between membrane enclosed organelles is an evolutionarily conserved feature of eukaryotes.

some of the major regulators of this process, illustrating their conserved function and stage of transport regulation [1].

Regulators of intermembrane flow

Coat proteins

Coat proteins form essential multimeric protein complexes that drive vesicle biogenesis from the donor compartment and are classified as either clathrin or non-clathrin coats (Figure 1.3) [2, 3]. Clathrin coats are the best understood coat complex, consisting of clathrin triskelions and adaptor proteins, and undergo polymerization that drives the formation of endocytic vesicles at the plasma membrane and certain Golgi-

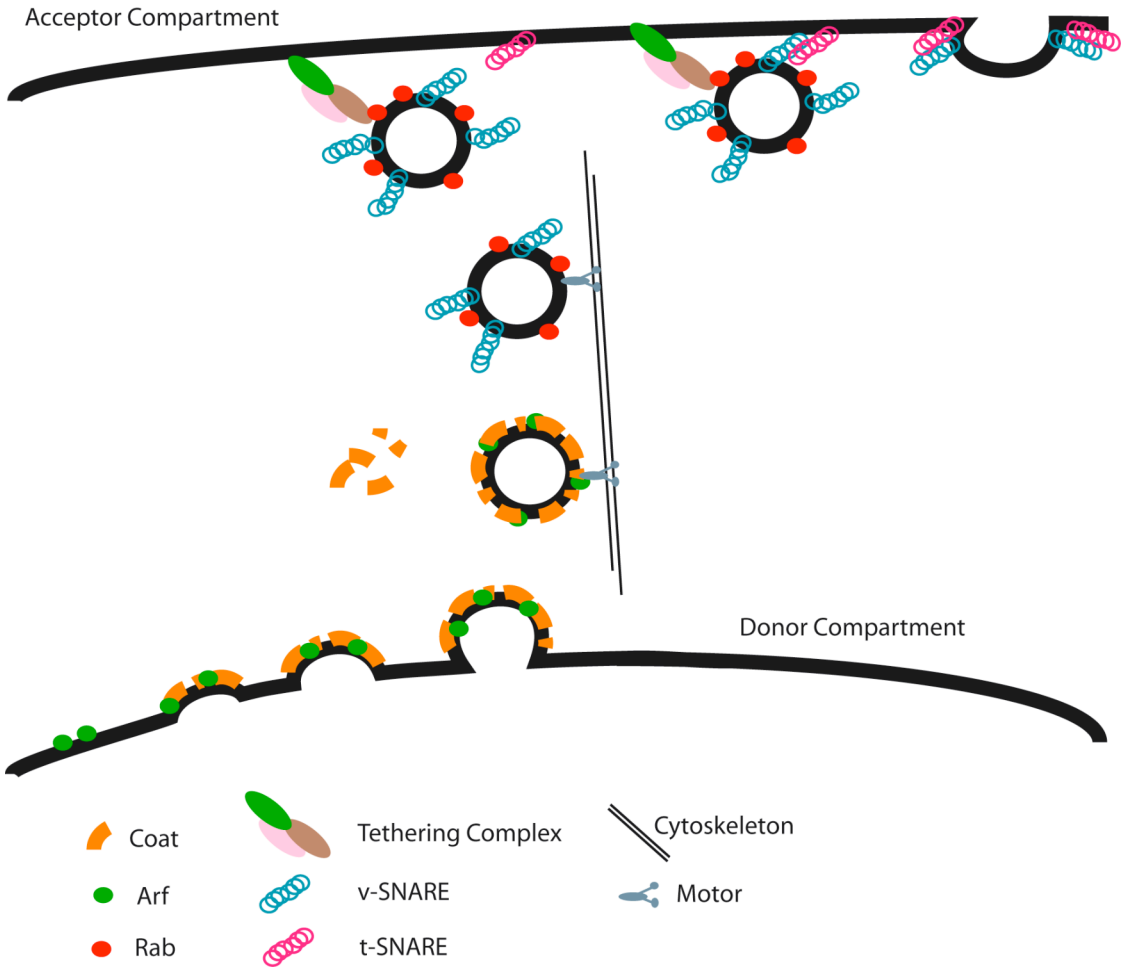

Figure 1.3. Major regulators of intramembrane transport and the stages they regulate. First, Arf GTPases are recruited to sites of budding where they recruit coat complexes which are one of the components required to generate the membrane curvature required for bud formation. Motor proteins transort the vesicle or organelle along the cytoskeleton to its acceptor compartment. Next, Rab GTPases interact with tethering complexes on the acceptor compartment. Finally, SNARE pairs form between v-SNAREs and t-SNAREs to drive membrane fusion.

derived vesicles [4]. First, adaptor proteins recruit cargo molecules and regulators of vesicle biogenesis, including the clathrin triskelion. Clathrin polymers then assemble to form an outer shell that coats the vesicle and aids in driving the membrane curvature required for vesicle formation and budding. After vesicle budding, the

vesicle uncoats and is transported to the acceptor compartment without its proteinaceous coat. Two well-characterized non-clathrin coated vesicles are COPI, also referred to as coatomer, and COPII vesicles that are involved in vesicle formation at the Golgi and ER, respectively.

COPI coats are found on Golgi-derived vesicles that travel in a retrograde direction toward the ER and are thought to have a role in recycling ER-resident proteins that may have been mistakenly transported to the Golgi [5]. A discussion of the functional analysis on COPI coat components in *S.cerevisiae,* Sec21p and Sec26p, will be presented in Chapter 2. Structural work from the Cerione laboratory indicated the γCOP C-terminus adopts a similar tertiary structure to the AP2 appendage domains that recruit factors that regulate clathrin coat formation [6]. Functional analysis suggests Sec21p requires its appendage domain for functions, providing insights into COPI vesicle formation [6]. COPII coats drive budding of endoplasmic reticulum (ER) derived vesicles en route to the Golgi apparatus, representing an initial stage of protein transport through the secretory pathway to the plasma membrane [7].

Monomeric guanosine triphosphate binding proteins

Two classes of small guanosine triphosphate (GTP) binding proteins, namely Arfs and Rabs, act as molecular switches to regulate vesicular transport, regulating discrete stages of trafficking [8]. Arf GTPases recruit specific coat components to the sites of vesicle budding and initiate vesicle formation (Figure 1.3). For example, Sar1 recruits the COPII coat to the ER membrane and Arf1 recruits the COPI coat to Golgi membranes. Rab GTPases function downstream of Arfs in transport and ensure proper targeting of vesicles and organelles to their acceptor compartment (Figure 1.3). For example, the yeast Rab Sec4p regulates targeting of vesicles derived from the

trans-Golgi Network (TGN) to the plasma membrane. Arf and Rab biology will be discussed in more detail in a later section.

Cytoskeleton and motor proteins

The cytoskeleton and cytoskeletal motor proteins form the framework for regulated organelle segregation and vesicular transport (Figure 1.3) [9, 10]. In *S.cerevisiae* actin polymers serve as tracks where myosin motors bind vesicles and organelles through adaptor molecules and use the energy stored in ATP to transport vesicles and organelles. In addition to the transport of vesicles and organelles, actin and myosin also play a role in transporting mRNA [11, 12]. In higher eukaryotes actin functions in regulating cell migration, cell shape and cell division. Microtubules, a second type of cytoskeleton, are critical for positioning the mitotic spindle, organelle transport and vesicular trafficking [13]. Microtubule-dependent motors kinesin and dynein bind to specific adaptor proteins that serve as a link to a particular organelle or vesicle, which can then be transported to different parts of the cell [14]. Adaptor proteins allow for a level of specificity and an increase in the types of molecules that can be transported.

Tethering complexes and SNAREs

Tethering complexes and SNAREs (soluble N-ethylmaleimide sensitive factor attachment receptor) ensure proper targeting and fusion of vesicles to their acceptor compartments (Figure 1.3) [1, 15-17]. Upon vesicle delivery, the tethering complex acts as a recognition module on the acceptor compartment identified by the Rab to deposit the vesicle or organelle, thereby ensuring a level of target specificity. Complexes with proposed tethering roles are found at the plasma membrane (exocyst complex), vacuole (HOPS (*h*omotypic fusion and vacuole *p*rotein *s*orting) complex),

and the Golgi (TRAPP (*Tra*nsport *p*rotein *p*article) and COG (*C*onserved *O*ctameric *C*omplex) complex). SNAREs are long coiled-coil proteins on the vesicle (v-SNARE) or the target compartment (t-SNARE) that trigger fusion of the vesicle membrane to the acceptor membrane. SNAREs have an underlying specificity in that particular v-SNAREs interact with specific t-SNAREs. Cognate SNARE pairing and specificity is aided by accessory factors, Sm/Sec1 proteins, that stimulate fusion [18]. Vesicle fusion occurs when the two membrane domains come into close contact and SNARE pairs are formed through the association of the joining coiled-coil domains. Together, SNARE pairs and tethering complexes allow for multilayered targeting specificity.

In summary, coat proteins and Arf GTPases regulate vesicle biogenesis, the cytoskeleton and cytoskeleton motors play critical roles in organelle and vesicle transport while Rab GTPases, tethering complexes, and SNARE proteins are essential for proper targeting and fusion events. These functions are conserved between *S.cerevisiae* and mammalian proteins. Functional conservation and its ease of genetic manipulation has enabled the use of yeast as a model system to study essential eukaryotic features, such as mechanisms of intracellular transport and organelle inheritance during cell division.

Organelle subdomain regulation

Although each organelle is a closed membrane system, studies have shown that they have the ability to organize into specialized functional subdomains. This is an additional evolutionarily conserved feature of eukaryotes and their intermembrane system, as it has been observed in yeast and mammals [19]. For example, the ER can be classified into different subdomains depending on the domain characteristics and function. When characterized based on organelle content, the ER has rough (ribosome-bound) and smooth (ribosome free) subdomains that have specialized

functions to insert newly synthesized transmembrane proteins into the ER lipid bilayer. When characterized based on shape, the ER has a tubular subdomain (high membrane curvature) and a sheet (low membrane curvature) subdomain and proteins have been identified that have differential affinity for specific domains [20]. Additionally, when characterized based on function, the transitional ER is a specialized subdomain that is a COPII budding hot spot [21].

The plasma membrane is another organelle that has an asymmetric distribution of contents and function [22-24]. Well-characterized subdomains in higher eukaryotes are the apical and basolateral subdomains which have specialized functions [25]. In *S.cerevisiae*, the plasma membrane of the daughter cell is enriched in proteins required for exocytic events. The ability of yeast to polarize its contents has allowed yeast to be used as a model to study cellular asymmetry. Chapter 8 focuses on an analysis of the requirements for the asymmetric localization of the ABC transporter Snq2p. The localization is intriguing as this transmembrane protein travels through the secretory pathway but is largely excluded from the regions of the plasma membrane where newly delivered membrane is deposited and appears to be localized away from sites of exocytosis.

Ras Superfamily of monomeric GTPases - molecular switches that control a variety of processes

The ras superfamily of small GTPases are evolutionarily conserved monomeric binary switches that regulate a diverse set of cellular processes (Figure 1.4). Members of the superfamily include Ras, Rho, Ran, Rab and Arf family members [26, 27]. The small GTPases display high structural conservation around the guanosine nucleotide-binding pocket and share a high affinity for GDP and GTP. The general fold adopted by the monomeric GTPases is a six-stranded β-sheet, with five parallel strands and

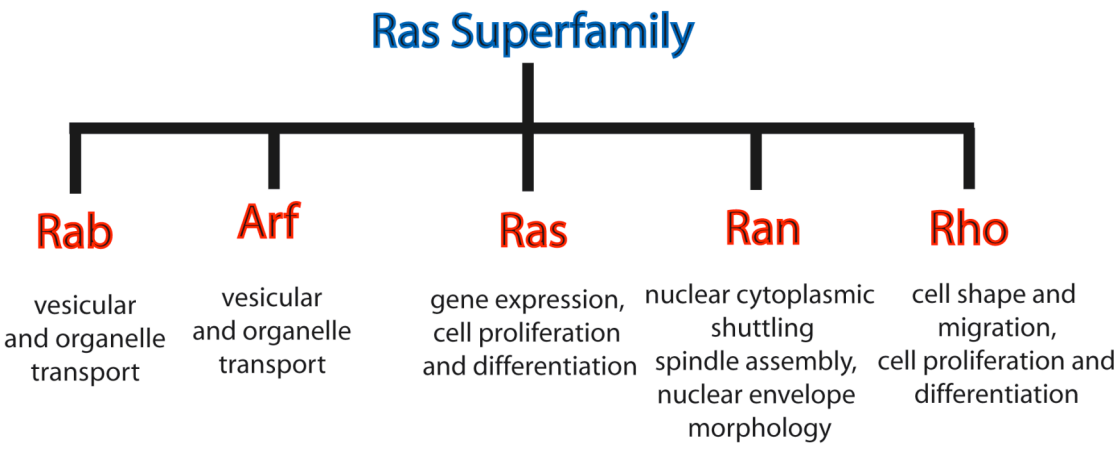

Figure 1.4. The Ras superfamily of small GTPases. Rab, Arf, Ras, Ran and Rho GTPases form the Ras superfamily and function as molecular switches that regulate a diverse array of cellular processes.

one antiparallel strand, flanked by five α-helices (Figure 1.5). The GTPases bind Mg^{++} that coordinates guanosine nucleotide binding. GTPases are referred to as molecular switches because they reside in two specific functional states, "on" or "off", dictated by the guanosine nucleotide bound (Figure 1.6).

The nature of the nucleotide bound state is highly regulated. When the GTPase is bound to GTP it is "on" and can interact with specific effector proteins and transmit signals (Figure 1.6). Conversely, when GDP is bound, the protein can no longer interact with effectors and is termed "off." The regulation of the "on" state is a critical feature of the GTPase to ensure that unregulated signals are not transmitted, as is found in cancers caused by oncogenic mutants of Ras [28, 29]. The GTPase relies on regulators, or guanine nucleotide exchange factors (GEF) to catalyze the release of GDP to allow GTP to bind. Ras family members generally have a slow intrinsic GTP hydolysis rate and rely on GTPase activating proteins (GAP) to stimulate GTP hydrolysis to turn the protein "off" and terminate the transmission of the signal.

Major conformational changes occur within two regions of the GTPase, designated switch I and switch II, to distinguish the "on" and "off" state. The switch

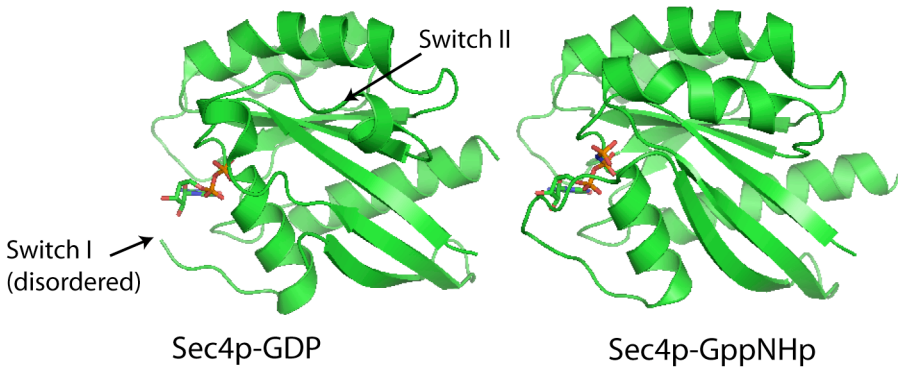

Figure 1.5. Nucleotide-dependent conformational changes. Switch I and Switch II are the regions of GTPases that display nucleotide-dependent conformational changes. These structural changes form the structural basis for GTPase-dependent regulation of cellular processes. Structures from [30].

regions are coordinated for effector binding in the GTP bound state. Figure 1.5 illustrates the structural differences between a GDP-bound and GTP-bound small GTPase. This particular structure is of the Rab GTPase Sec4p, showing that Switch I is flexible and disordered when GDP is bound. Upon GTP binding, this region is ordered into a loop in proximity to the guanine nucleotide-binding pocket. Switch II is primarily a loop when the GTPase is GDP bound and becomes an alpha helix when GTP binds [30]. These nucleotide-dependent conformational changes form the structural basis for regulating GTPase-effector binding events and therefore control the GTPase-dependent signaling processes.

Spatial regulation is an additional level of GTPase biological activity regulation [26, 27]. Many members of the Ras superfamily are post-translationally modified with prenyl attachments on cysteine residues that allow for membrane insertion. The majority of Ras and Rho family members have a CAAX motif at the extreme C-terminus, where C is cysteine, A is aliphatic amino acid and X is any amino acid. The prenyl moiety is generally either a farnesyl (15 carbon) or geranylgeranyl

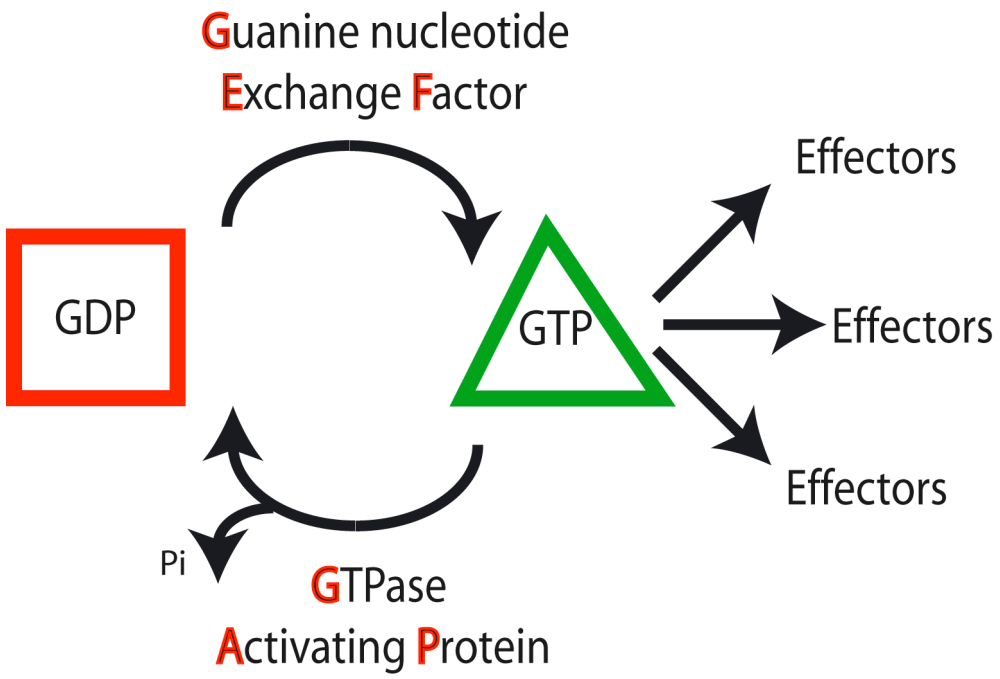

Figure 1.6. The GTPase activation cycle. GTPase activation is regulated by a nucleotide-dependent mechanism. Guanine nucleotide exchange factors stimulate GDP for GTP exchange, thereby activating the GTPase. GTPase activating proteins stimulate GTP hydrolysis to turn the GTPase off. When GTP-bound, the GTPase can interact with downstream effectors.

(20 carbon) group [31]. The majority of Rab GTPases contain two cysteine residues that allow for dual prenylation [32]. The CAAX motif, or di-cysteine motif found on Rab proteins, is a consensus sequence for farnesyl transferase or geranylgeranyl transferase enzymes that catalyze the attachment of the lipid group to the protein.

Spatial regulation of the small GTPase is critical for function. For example, Ras requires lipid modification to be positioned at the plasma membrane where it can transmit signals. Further support for the importance of this regulation is demonstrated by a class of anticancer agents, farnesyl transferase inhibitors, which target this requirement for Ras function in the hope to diminish the signaling capacity of the hyperactive oncogenic forms of Ras found in many cancers [33].

Ras GTPases

The Ras oncogene is the founding member of the superfamily [26, 27]. It serves as a signaling hub where activated Ras signals to a set of effectors that regulate gene expression, cell proliferation and differentiation. About 30% of all human tumors contain a hyperactive *ras* mutation that signals constitutively to promote cell proliferation. This is a classic example of how unregulated signaling at the molecular level can lead to drastic physiological changes. Members of the Ras family include H-Ras, K-Ras, RalA and RalB. Ras is the best characterized small GTPase in the superfamily. The GEF SOS (*S*on *o*f *s*evenless) stimulates GTP binding of Ras and leads to the activation of numerous signaling cascades, including the MAP-kinase (*m*itogen-*a*ctivated *p*rotein kinase) phosphorylation cascade. The MAP-kinase cascade leads to the phosphorylation of proteins that influence changes in gene expression leading to cell differentiation and growth [34].

Rho GTPases

Members of the Rho family regulate a range of cellular processes. They have a diverse set of GEFs and GAPs that funnel signals to regulate gene expression, actin organization, cell polarity and cell cycle [35]. RhoA, Rac1 and Cdc42 are the best characterized Rho family members. Rho proteins influence cell migration, cell shape and cell polarity through its regulation of actin polymerization. Rho proteins contain a single geranylgeranyl attachment on their C-terminus that allows for membrane insertion, however, it can be found in two populations: membrane-bound or cytoplasmic [31]. The 20-carbon lipid moiety is extremely hydrophobic and therefore requires Rho proteins, when bound to GDP, to form a heterodimer with Rho GDI (GDP dissociation inhibitor). Rho GDI extracts Rho from membranes shielding the

hydrophobic moiety from the aqueous cytosol environment [36]. This serves as one of the mechanisms to transport Rho proteins between intracellular compartments.

Ran GTPases

Ran GTPases regulate nuclear-cytoplasmic shuttling of RNA and proteins [26]. Ran proteins are soluble and do not have lipid modifications. Concentration gradients exist with a high Ran-GTP concentration in the nucleus and high Ran-GDP concentration in the cytoplasm. This occurs through the spatial regulation of its GEF (nuclear) and GAP (cytoplasm), which is crucial for Ran-dependent shuttling processes. Importins recruit cargo in the cytoplasm where active Ran is in low abundance. Once in the nucleus, Ran-GTP dissociates the importin from cargo and allows importin to translocate back into the cytoplasm for additional rounds of nuclear transport. Exportins actively transport molecules from the nucleus to the cytoplasm. A high nuclear concentration of Ran-GTP concentration allows exportins to bind cargo and translocate to the cytoplasm. Once exportins are in a low Ran-GTP environment, cargo is released. In addition to the nuclear-cytoplasmic shuttling of cargo, Ran regulates spindle assembly, DNA replication and nuclear envelope assembly [37].

Arf GTPases

Arf proteins regulate vesicular transport events, particularly cargo sorting and vesicle formation, by recruiting coat complexes [38, 39]. The nucleotide-bound state of Arf dictates is location in the cell. Arf proteins are post-translationally modified with a myristoyl moiety at the N-terminus. Arfs can be membrane-bound or soluble in the cytosol, however, the biological activity of Arf only occurs on membranes. When GDP-bound, the myristoyl group is inside the core of the protein and shielded from the

cytosol. Therefore, unlike Rho and Rabs, Arfs do not require a GDI-like molecule to keep the protein soluble in an aqueous environment. Upon nucleotide exchange, conformational changes expose the lipid moiety that allows for membrane insertion. Thus, Arf exchange factors dictate the location of Arf membrane recruitment and activity and vesicle biogenesis. Activated Arf proteins then recruit coat complexes to initiate vesicle budding. Arf1 and Arf6 are the best characterized Arf GTPases. Arf1 recruits COPI coat proteins at the Golgi and influences vesicle budding from the Golgi, while Arf6 is involved in clathrin coat recruitment and endocytic budding from the cell surface.

Rab GTPases

Rab GTPases, numerically the largest family of the Ras superfamily of small monomeric GTPases, are regulators of vesicle and organelle transport, localizing on the cytosolic leaflet of membranes to regulate discrete targeting steps [15, 38]. Rab proteins recruit effector proteins to ensure proper vesicle and organelle targeting. There are 11 Rabs encoded in the *S.cerevisiae* genome and more than 60 Rabs encoded in the human genome, with significant conservation from yeast to mammals. Rab family proteins have diverse intracellular localizations reflecting the stage of vesicular transport that they regulate [15]. Chapter 3 focuses on studies using GFP-tagged Rab proteins in *S.cerevisiae* to investigate their specific intracellular distribution in cells [40]. Rabs can be found in two environments: membrane bound or soluble in the cytosol (Figure 1.7). The majority of Rabs have two geranylgeranyl lipid moieties attached to their C-terminus which allows for association with the membrane. Similar to Rho GTPases, Rabs require heterodimerization with Rab GDI to be extracted from membranes and remain soluble in the aqueous cytosolic environment. Rab GDI is essential for recycling Rab proteins from their acceptor

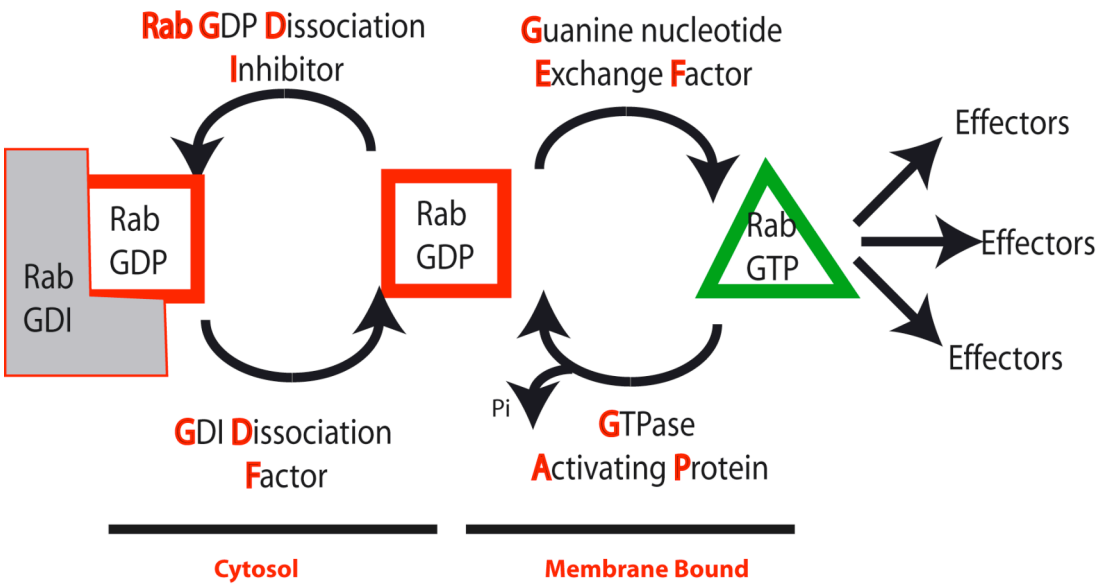

Figure 1.7. Rab guanine nucleotide cycle. Rab GTPase function is regulated by a GTP and GDP-bound activation cycle similar to all GTPases. The Rab nucleotide state also determines the cellular environment of the Rab. When GDP-bound, it can be either membrane bound or cytosolic. Rab GDI extracts the Rab from membranes and forms a cytosolic heterodimer. When bound to GDI, the Rab is unable to be activated by a GEF which can only happen upon dissociation from GDI and insertion into the membrane. This serves as a spatial regulatory mechanism for Rab activation.

compartment back to the donor compartment to allow for additional rounds of transport regulation.

Sec4p is a central regulator of polarized exocytosis

The *S.cerevisiae* genome encodes 11 Rabs, each localizing to distinct organelles that reflect the stage of membrane transport they regulate (Figure 1.8). For example, Ypt1p localizes to the Golgi where it regulates Golgi vesicle trafficking events [41]. Sec4p is the Rab GTPase that regulates the targeting of vesicles that allow for polarized growth [42]. Exocytic vesicles from the trans-Golgi network (TGN) travel along actin cables in a Myo2p-dependent manner where they fuse with the plasma membrane [43, 44].

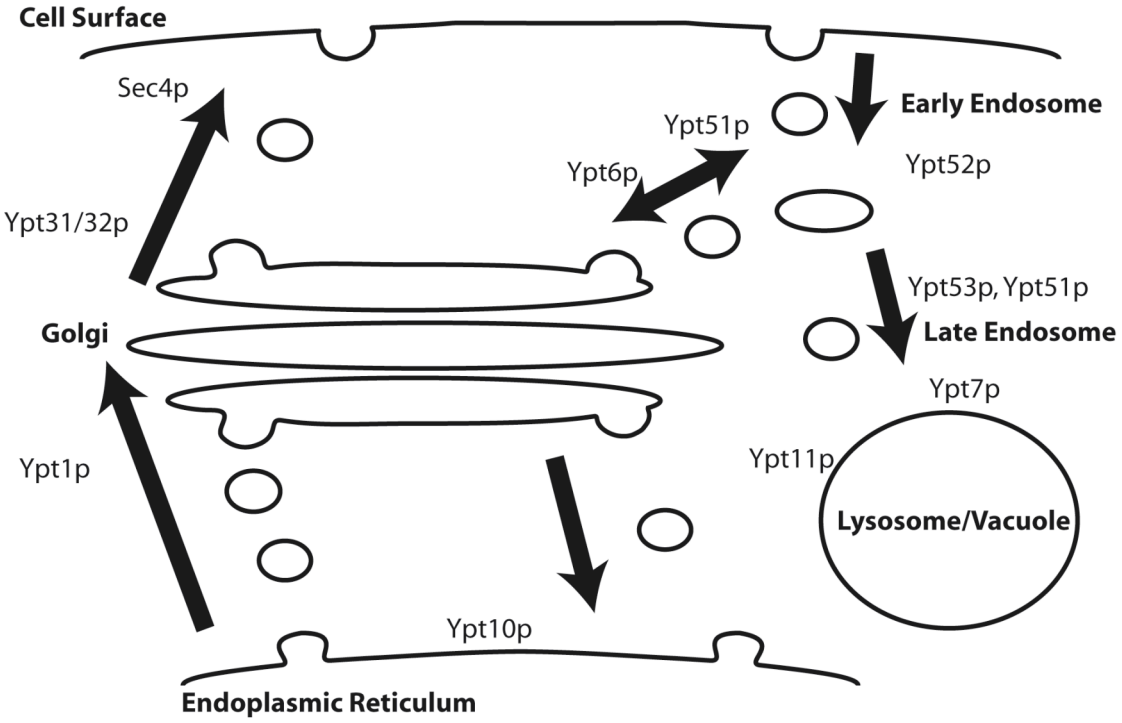

Figure 1.8. *S.cerevisiae* Rab-dependent regulation of intracellular transport. Rab GTPases regulate distinct stages of transport.

Sec4p is an essential protein in yeast as loss of function is lethal and causes accumulation of post-Golgi vesicles [45]. It has orthologs in higher eukaryotes, including the mammalian rab3a and rab8 [46]. The GEF Sec2p regulates its activity, stimulating the release of GDP, stabilizing the nucleotide-free state and allowing GTP to bind. Sec2p has specific exchange activity for Sec4p and cannot stimulate nucleotide exchange on other Rabs. Sec2p is an essential protein in yeast and is orthologous to the mammalian Rab exchange factor Rabin 8 [47].

Dss4p (*D*ominant *S*uppressor of *Sec4*) also influences the Sec4p nucleotide bound state [48]. It is a nonessential protein that is found both on membranes and in the cytosol. Dss4p stimulates the release of both GTP and GDP from Sec4p and displays guanine nucleotide dissociation stimulator features. It also displays this property towards the Golgi Rab Ypt1p.

GAPs stimulate the hydrolysis of GTP to GDP to turn off Sec4p. Multiple proteins have been shown *in vitro* to possess GAP activity on Sec4p, including Gyp1p, Gyp2p, Gyp5p, Gyp6p, Gyp7p, Gyl1p, Msb3p and Msb4p [49-52]. These proteins also possess GAP activity *in vitro* on other Rab proteins, indicating there is promiscuity in this function. More importantly, Msb3p, Msb4p, Gyp5p and Gyl1p have *in vivo* functions in regulating exocytosis determined through localization studies and mutational analysis [49, 50].

The exocyst complex is an octameric effector complex that ensures correct targeting of secretory vesicles [53]. GTP-bound Sec4p interacts with the exocyst subunit Sec15p. The other exocyst subunits (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Exo70p and Exo84p) form the remaining complex to target secretory vesicles to the plasma membrane (Figure 1.9) [53]. GTP-bound Sec4p also interacts with another vesicle-targeting module and forms a ternary complex with Sro7p and the plasma membrane t-SNARE Sec9p [16, 54]. Sec4p activation and effector binding events are critical focal points in exocytosis regulation. Mutational analysis has found that loss of function of these proteins generally results in an accumulation of secretory vesicles due to a failure of vesicle delivery or fusion.

Regulators of post-Golgi vesicle transport

Sec4p structure and function

Sec4p is one of the central regulators of vesicular transport from the TGN to the plasma membrane. Sec4p has a tertiary structure similar to other Ras superfamily members centered around the guanine nucleotide-binding pocket but differs at Rab family and subfamily domain regions that differentiate the Rab family from other members of the Ras superfamily [30].

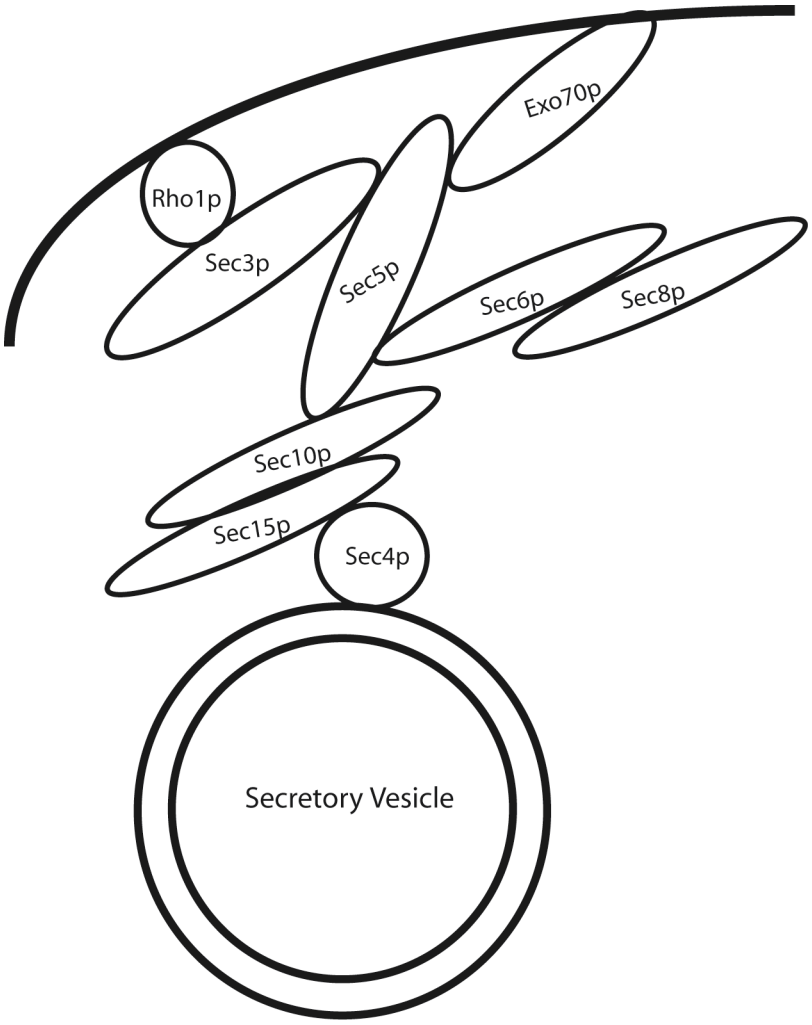

Figure 1.9. Exocyst assembly to tether secretory vesicles to the plasma membrane. A schematic representation of exocyst assembly suggesting how it forms a proposed tethering complex to target secretory vesicles to the plasma membrane in a variety of cell types. This particular depiction is taken from yeast biochemical interaction data and based on the work of [53].

Rab family motifs are distinct regions in the GTPase that make Rabs structurally and functionally unique from other small GTPases. These regions are thought to add higher levels of specificity for Rabs and their binding partners [55, 56]. As discussed earlier, Sec4p undergoes nucleotide-dependent conformational changes at their switch regions. Among the Rab family motifs, the switch regions consist of motif 1, motif 3, and motif 4. For Sec4p, the loop2 region has been termed the

effector-binding domain as this region was shown to be necessary, but not sufficient, for binding to its effector Sec15p using yeast two hybrid analysis [53]. Sec4p chimeric studies revealed that the effector domain is critical for Sec4p function *in vivo* as substituting the *YPT1* effector domain was unable to complement a deletion of *SEC4* [57, 58]. The C-terminal hypervariable domain is a highly divergent region among Rab proteins and therefore was proposed to be the determinant that specifies localization [59]. Experimental data, however, have shown that this region is not the sole determining region for localization and additional domains also influence localization [57, 58].

Rabs appear to use the switch regions and Rab family motifs to regulate interactions with binding proteins. Although Sec4p may use the Rab family motifs for protein-protein interactions, it is still solely the switch regions that undergo conformational changes dictated by nucleotide binding, as was described earlier and depicted in Figure 1.5 [30]. The Rab subfamily domains appear to also influence Rab-effector interactions. Three of the subfamily motifs were found to reside on the Rab3a-Rabphilin interaction face [60].

Exocytosis requires (1) vesicle transport and recruitment to the plasma membrane, (2) vesicle tethering to the plasma membrane and (3) vesicle fusion with the plasma membrane. A number of proteins have been identified as regulators of post-Golgi vesicle transport through a variety of genetic and biochemical studies. The role of Sec2p, Sec4p, the exocyst, Sro7p, Rho1p, Snc1/2p, Sso1/2p, Sec9p, and RabGDI in exocytosis has been characterized. Generally, loss of function of any of these proteins results in a disruption of exocytosis and an accumulation of vesicles, indicating their requirement for the promotion of exocytosis. These yeast proteins have proven to contain essential functions to maintain cell growth and have functional equivalents in higher eukaryotes (Figure 1.10).

Function	*S.cerevisiae*	Higher Eukaryotes
Rab GTPase - vescile targeting at the plasma membrane	Sec4p	Rab3, Rab8
Rab guanine nucleotide exchange factor	Sec2p	Rabin 8
Vesicle docking and SNARE binding	Sec1p	Sec1
Exocyst Complex subunit - vesicle tethering	Sec3p	Sec3
Exocyst Complex subunit - vesicle tethering	Sec5p	Sec5
Exocyst Complex subunit - vesicle tethering	Sec6p	Sec6
Exocyst Complex subunit - vesicle tethering	Sec8p	Sec8
Exocyst Complex subunit - vesicle tethering	Sec10p	Sec10
Exocyst Complex subunit - vesicle tethering	Sec15p	Sec15
Exocyst Complex subunit - vesicle tethering	Exo70p	Exo70
Exocyst Complex subunit - vesicle tethering	Exo84p	Exo84
SNARE pair dissociation	Sec18p	NSF
Rab GTPase recycling	Sec19p	Rab GDI
Plasma membrane t-SNARE protein, vesicle fusion	Sec9p	SNAP-25
Actin-based motor that moves secretory vesicles	Myo2p	MyosinV
Actin cytoskeleton component	Act1p	Actin
Plasma membrane t-SNARE protein, vesicle fusion	Sso1/2p	Syntaxin
Secretory vesicle v-SNARE, vesicle - plasma membrane fusion	Snc1/2p	Synaptobrevin
Polarity establishment	Rho3p	Rho GTPases
Secretory vesicle docking and fusion	Sro7p	Lethal Giant Larvae
Secretory vesicle docking and fusion	Sro77p	Lethal Giant Larvae

Figure 1.10. Conservation of regulators of polarized exocytosis.

Sec2p structure and function

Sec2p catalyzes the activation step of Sec4p, leading to GTP-bound Sec4p. Once activated, Sec4p can interact with downstream effectors to target vesicle delivery. Sec2p and Sec4p are both essential in yeast and localize in a polarized distribution to the sites of exocytosis at the bud tip (Figure 1.11). Sec4p activation by Sec2p requires the N-terminal coiled-coil domain on Sec2p, which is responsible for homodimerization (Figure 1.11) [47, 61]. *sec2-59* is a temperature sensitive allele of *SEC2* that contains a truncation caused by a stop codon insertion after residue 374. This mutant has exchange activity equivalent to that of wildtype Sec2p *in vitro* but is

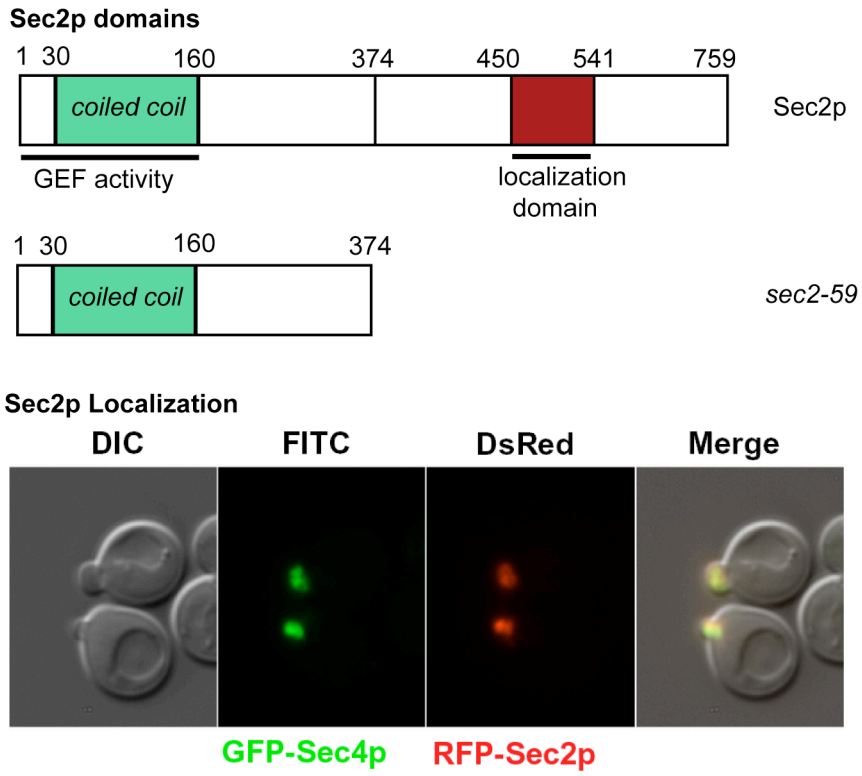

Figure 1.11. Sec2p domains and localization. Sec2p uses its coiled-coil domain for homodimerization. The first 160 residues are also where Sec2p interacts with Sec4p and possesses its exchange activity. Residues 450-541 have been shown to be essential, but not sufficient, for localization and therefore has been termed the localization domain. The Sec2p mutant allele, *sec2-59*, is a truncated version of the protein that is deficient in localization but *in vitro* has equivalent exchange activity to wildtype Sec2p. Sec2p andSec4p localize in a polarized distribution in cells in the bud tip.

mislocalized in cells [47], indicating the exchange activity domain is within the first 374 residues. Through further analysis of the residues critical for exchange, the first 160 residues of Sec2p were shown to interact with Sec4p and are sufficient for exchange activity [47].

Structural insights into the mechanism by which Sec2p stimulates Sec4p activation have just recently been reported with an x-ray crystal structure of the complex formed by Sec2p (residues 17-167) and the nucleotide-free form of Sec4p

(residues 18-187) [62]. Two Sec2p molecules form a parallel coiled-coil domain that causes major conformational changes of the switch regions and the nucleotide-binding pocket and catalyzes nucleotide release and stabilization of the nucleotide-free form of Sec4p upon binding. Sec2p binding orders Switch I, which is normally disordered in GDP-bound Sec4p. Sec2p has high specific activity for Sec4p and cannot exchange nucleotides bound to Ypt1p. However, Sec2p does exchange nucleotides on a Ypt1p chimera that has the Sec4p Switch I domain, indicating the Switch I region is the primary determinant for Sec2p exchange activity. The residues on Sec2p that are important for exchange and interaction with Sec4p are highly conserved with Rabin 8, another coiled-coil GEF that regulates exocytosis [47].

Both Sec2p and Sec4p associate with membranes. As discussed earlier, Sec4p associates with membranes through dual prenylation at the C-terminus [63]. The mechanism by which Sec2p associates with membranes is not understood. It can be found in the cytoplasm and associated with membranes [64]. Sec2p does not contain a transmembrane domain or lipid modifications to attach it to membranes, however, the C-terminus is responsible for the association between Sec2p and secretory vesicles [64]. The Sec2p domain consisting of residues 450-541 is required, but not sufficient, for localization. The truncated *sec2-41* mutant (residues 1-450) is mislocalized in cells while a truncated *sec2 (1-541)* is on membranes and localizes to sites of polarized secretion [64].

Sec2p has also been found to interact with exocyst subunit Sec15p and the Golgi Rab Ypt32p [65, 66]. A current model suggests that Ypt32p recruits Sec2p to secretory vesicles and the binding of Sec15p displaces Ypt32p from Sec2p so that Sec2p can activate Sec4p. It is hypothesized that vesicle tethering triggers the release of Sec2p from the vesicle so it can regulate additional rounds of post-Golgi transport

and that constant recycling of Sec2p is required to maintain its polarized localization [66].

Exocyst structure and function

To date two Sec4p effectors have been identified: Sec15p and Sro7p [16, 53]. These two proteins have been shown to preferentially bind GTP-bound Sec4p. Sec15p is a subunit of the exocyst complex. Sec15p interacts with Sec10p that then interacts with the rest of the exocyst complex that has been proposed to serve as a platform to tether vesicles to the plasma membrane (Figure 1.9) [53]. Exocyst function is not required for the delivery of secretory vesicles to the plasma membrane, but when exocyst function is lost, vesicles fail to fuse with the plasma membrane. The exocyst complex serves as a scaffold complex to tether secretory vesicles to sites of polarized exocytosis at the plasma membrane.

A current model for exocyst localization suggests that the majority of exocyst subunits associate with secretory vesicles prior to vesicle tethering, with the exception of Sec3p and Exo84p, and that complex formation occurs on the vesicle itself [67]. Sec3p has been suggested to act as the polarity landmark to recruit an assembled exocyst at polarized sites, when reports indicated Sec3p localizes independent of the other exocyst subunits, however, conflicting data has been presented to suggest otherwise [67-69].

The exocyst complex is a conserved octameric tethering complex that in higher eukaryotes functions in a variety of cell types. Reports have emerged that have begun to describe the exocyst's function in higher eukaryotes in a variety of cell types. From this work, it is apparent that the exocyst has a crucial function in regulating vesicular targeting and tethering to control cell growth and development and membrane expansion and asymmetry.

Studies in *Drosophila* indicate exocyst function is more complex than initially thought [70-77]. Mutations in the *sec5* and *sec6* subunits disrupt lipid and membrane protein insertion in neuronal plasma membranes while synaptic transmission remains unaffected [73, 75]. This effect is not solely neuronal, as *sec6* mutations lead to defects in membrane insertion in photoreceptor cells and *sec5* function is required for plasma membrane trafficking and polarity establishment in *Drosophila* ovaries [74, 76]. Adding to the complexity of the exocyst's role in higher eukaryotes was a study describing mutations in *sec15* in *Drosophila* photoreceptors. This report found that *sec15* function is not required for photoreceptor survival or axonal extensions, indicating *sec15* is not required for membrane insertion. However, *sec15* is required to identify appropriate synaptic partners [77]. It is intriguing to speculate why mutations in different subunits gives rise to different phenotypes. One possible scenario could be explained by the existence of different subcomplexes of exocyst components that are recruited to specific domains. Sec15 and Sec8 have been reported to colocalize with Sec5, but not completely, in developing *Drosophila* lamina, suggesting different components may carry out different roles [77].

In mammals, the exocyst targets vesicles to particular domains of the plasma membrane and is involved in extending neuronal axes, sites of synapse formation, and the maintenance of the basolateral domain in polarized epithelial cells. Different signaling pathways may regulate exocyst assembly or function. For example, Sec5 and Exo84 were shown to be direct effectors of the small GTPase Ral [71, 72]. This interaction implicates exocyst function and defects in polarity establishment and maintenance with tumor progression and apoptosis, as Ral is a central signaling component whose function is misregulated in certain tumors.

In *S.cerevisiae*, exocyst function influences other processes in addition to vesicle tethering. Exocyst subunit Sec3p is required for ER inheritance in the

daughter cell during cell division. A *sec3Δ* strain exhibits defects of cortical ER inheritance, while Golgi and mitochondria inheritance remains normal, suggesting Sec3p and the exocyst complex is required for tethering the ER to the growing daughter cell plasma membrane [78]. Also, data suggest a functional link between exocyst function and the ER translocon apparatus – thereby linking exocytosis (the late stage of secretion) to protein synthesis (the initial stage of secretion). The translocon subunits *SEB1, SEC61,* and *SSS1* were identified as multicopy suppressors of exocyst mutants [79]. Furthermore, the β-subunit of the translocon, *SEB1,* associates with exocyst subunits Sec8p, Sec15p and the Rab Sec4p.

Initial indications of the three-dimensional exocyst structure first emerged using quick-freeze/deep etch electron microscopy (EM) on the mammalian exocyst complex. The overall architecture presented in this study suggested the exocyst adopts a shape of an elongated "body" with "arms" radiating from the top of the body [80]. The three dimensional structures have been determined for domains of four exocyst subunits. Structures of the C-terminal domains of Sec15, Exo84, and Sec6 and the full length Exo70p have been solved to atomic resolution (Figure 1.12) [81-85]. All four structures reveal a common topology: elongated rods formed from alpha helical bundles. The remaining exocyst subunits also have predicted alpha helical secondary structures, suggesting that an elongated rod conformation may be a common topology of the complex. The assembled elongated rods may adopt a scaffold similar to the overall structure observed by EM where subunits can stably associate to form the octameric complex, or be found *in vivo* as a set of subcomplexes that take imputs from other signaling pathways to regulate overall assembly.

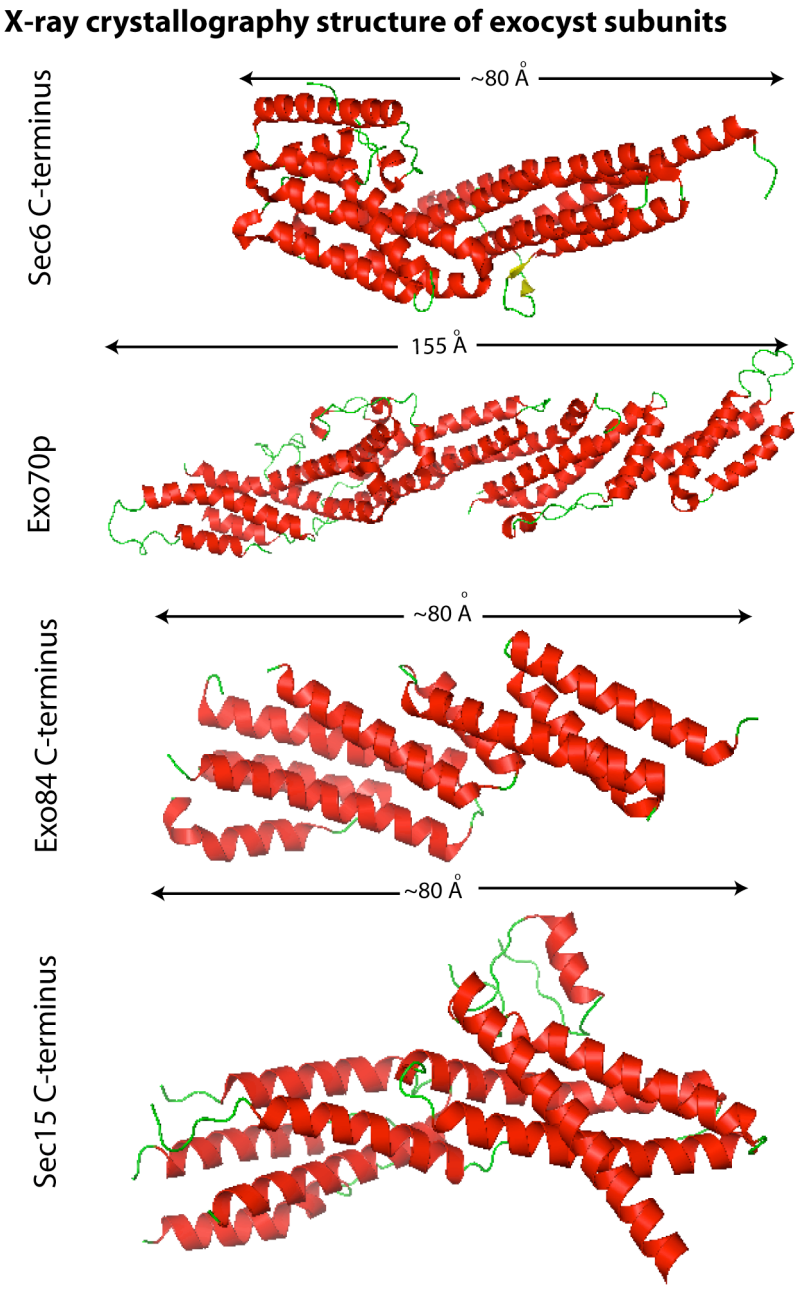

Figure 1.12. Structural analysis of exocyst complex. X-ray crystallography structures of exocyst subunits or subunit domains, illustrating a common theme in exocyst subunit structure: elongated rods generated from helical bundles. α-helices are shown in red, loops are shown in green and β-sheets are shown in yellow. Structures are from [84] (Sec15), [82] (Exo84), [83] (Exo70), and [81] (Sec6).

Sro7p

Sro7p is a member of the lethal giant larvae (lgl) tumor suppressor family and was shown to preferentially interact with GTP-bound Sec4p [16, 86, 87]. Grosshans *et al* showed that Sro7p partially shares function with the exocyst complex, however, the exocyst and Sro7p also have different functions [16]. Therefore, Sec4p targets secretory vesicles to the plasma membrane through interacting with at least two independent components. Interestingly, the interaction of Sec4p with Sro7p results in a ternary complex with the exocytic t-SNARE Sec9p [16]. Through these two effector interactions Sec4p can potentially regulate vesicle tethering, through its interaction with the exocyst complex, and vesicle fusion, through recruiting Sro7p and the t-SNARE Sec9p.

Exocytic SNAREs

SNARE proteins catalyze fusion events between lipid bilayers. SNAREs adopt an elongated coiled-coil tertiary structure and are inserted into membranes via a transmembrane domain or lipid modification. These proteins ensure a level of transport specificity. The exocytic v-SNAREs Snc1p and Snc2p interact preferentially with t-SNAREs on the plasma membrane Sec9p, Sso1p, and Sso2p, as regulated by the Sm/Sec1 proteins [18, 88-90]. Interactions between the SNARE pairs drive the vesicle and target membranes to come in close proximity and stimulate membrane fusion (Figure 1.3). Snc1p and Snc2p are highly homologous and have redundant functions *in vivo* and are homologous to synaptobrevin, a v-SNARE found in higher eukaryotes. Deletion of either single protein results in minimal phenotypes, however deletion of both proteins results in an inviable cell. Sso1p and Sso2p are functionally conserved to the mammalian t-SNARE syntaxin (Figure 1.10). Sso1p and Sso2p have similar redundant characteristics.

Rab GDI

A single open reading frame (ORF), *SEC19*, encodes the Rab GDI protein in the yeast genome. It is an essential gene in yeast and was isolated as a mutant allele that accumulates intracellular membranes when shifted to restrictive conditions [45]. Unlike the proteins described above that are thought to function solely in the exocytic stage of transport, RabGDI regulates all stages of Rab-dependent transport. This is indicated from mutant analysis where *SEC19* mutants accumulate all intracellular membranes, including vesicles, ER, Golgi, and endocytic membranes [32].

RabGDI function is conserved throughout evolution and is critical for homeostasis in humans. Mutation of *α-RabGDI* at residue 92, I to P, leads to X-linked mental nonsyndromic mental retardation [91]. GDI extracts GDP-bound Rabs from membranes and serves two functions: to negatively regulate Rab function through inhibiting GDP for GTP exchange and to recycle Rab from its acceptor compartment back to its donor compartment to allow for additional rounds of transport regulation (Figure 1.7).

Because there is only one Rab GDI locus in *S.cerevisiae*, it can interact with all 11 Rabs. Structural characterization between the Rab-Rab GDI interaction of mono-geranylgeranylated Ypt1p and Rab GDI indicate that that GDI contacts the Rab at three major regions: switch I and switch II and the C-terminus of Ypt1p [92]. Interactions with the switch I and switch II regions ensure GDI extracts only GDP-bound Rab from the membrane. The interaction with the C-terminus is mainly through hydrophobic contacts that eliminate a requirement for side chain specificity. This is crucial because one GDI molecule is available to heterodimerize with all 11 Rab molecules and the C-terminus is a highly divergent region of the protein.

Rab cascades to regulate vectoral transport

An emerging theme in regulation of membrane transport is the idea of Rab cascades to recruit factors to direct transport steps in the secretory pathway. This concept first came to light in yeast with Golgi to plasma membrane vesicle transport. In a linear depiction of the Rabs that regulate the secretory pathway, Ypt1p functions at the Golgi, Ypt31p and Ypt32p regulate late Golgi transport and Sec4p controls trafficking of post-Golgi vesicles (Figure 1.8).

The TRAPP complex is a putative Ypt1p effector protein and stimulates GDP for GTP exchange on Ypt32p [93, 94]. Ypt32p was shown to interact with Sec2p in a GTP-dependent manner where Sec2p is a Ypt32p effector [65]. Under this signaling cascade, a vesicle containing GTP-bound Ypt1p would recruit the TRAPP complex to the vesicle. The exchange factor would then recruit Ypt32p to the vesicle and allow Ypt32p to direct the transport through the late-Golgi apparatus. Ypt32p then recruits Sec2p to the vesicle where Sec2p recruits and activates Sec4p on the vesicle. GTP-bound Sec4p then targets the vesicle to the plasma membrane for polarized secretion. Therefore, through recruitment of different Rabs, effectors and GEFs, a vesicle could potentially change its identity from a Golgi vesicle to a secretory vesicle.

This scenario also takes place in higher eukaryotes. Rink *et al* described a pathway utilizing Rab5 and Rab7 to control endosomal trafficking [95]. Their study suggests that early endosomes are decorated with Rab5 and abruptly change to Rab7-containing endosomes. In this process, Rab5 recruits a Rab7 GEF to the vesicle that recruits Rab7. This suggests a mechanism where vesicle identity is changed from an early endosome to a late endosome through the recruitment of different molecules to the vesicle surface.

Regulators of post-Golgi transport

The proteins described above (with the exception of Rab GDI) positively influence exocytosis: their function promotes exocytosis and loss of function blocks exocytosis. Cellular processes generally have positive and negative regulatory components. As described above, many positive regulators of exocytosis have been characterized, however, little is known about negative regulators of this process. Our recent work has identified proteins that function to negatively regulate exocytosis and removal of these factors results in an increase in exocytic function. In Chapters 4 and 5, I will discuss work I have done that identified the Elongator Complex as a regulator of exocytosis [96]. In Chapters 5 and 6, I will discuss additional work to further characterize this regulation and the identification of other proteins that are involved in this pathway.

The Elongator Complex has functional implications in multiple cellular processes

The Elongator Complex is a six-subunit complex that was initially identified through copurifying with the hyperphosphorylated form of RNA polymerase II (PolII) [97]. Three of the six subunits (Elp1p, Elp2p and Elp3p) were first identified to interact with the elongating form of RNA PolII. Two reports followed that identified three additional polypeptides that associate with Elongator: Elp4p, Elp5p and Elp6p [98, 99].

The holo-Elongator complex can be biochemically purified as two subcomplexes consisting of the three large subunits, Elp1p-Elp3p, and the three small subunits, Elp4p-Elp6p, under high salt conditions [98, 99]. Primary sequence analysis predicts Elp3p to possess two catalytic domains: an acetyltransferase domain and a SAM (*S-a*denosyl*m*ethionine) radical domain [100, 101]. Acetyltransferase domains attach acetyl groups to substrate side chains using acetyl-CoA. The acetylation

modification changes the microenvironment of that residue and can impact protein function. Acetylation is a well established post-translational modification in the nucleus that effects transcription of specific genes, however, the idea that acetylation also plays an important regulatory role in the cytoplasm has only recently been appreciated [102]. Specifically, this was observed in MEK2 and IκB kinase proteins, where acetylation inhibits the kinase activity of these cytosolic resident proteins [103]. The function of the Elp3p SAM radical domain is less definitive, as it was recently identified [101]. SAM radical domains use S-adenosylmethionine to catalyze a number of reactions including methylation and protein radical formation. Elp1p and Elp2p appear to be scaffold proteins based on primary sequence homology predictions, containing multiple WD-40 motifs, which are involved in protein-protein interactions [104]. Sequence analysis of Elp4p, Elp5p, and Elp6p yields less insight into possible functions. Elp4p has weak homology to a RecA-like domain but the true architecture of the protein remains to be determined experimentally.

The holo-Elongator three-dimensional structure has yet to be determined but Elp1p was found to be essential to maintain holo-Elongator structural integrity and loss of Elp1p results in dissociation of the two subcomplexes. Also, Elp4p and Elp6p are required to maintain an interaction between Elp5p and Elp2p, suggesting these may form a bridge connecting these two proteins [105, 106].

Biochemical analysis found that Elp3p requires all five subunits to maintain catalytic activity, suggesting that complex biochemical activity may reside in the Elp3p subunit while the other five subunits may regulate its function [100, 107]. Phenotypic data supports this since deletion of any subunit generally gives rise to the same phenotype [100, 105, 106].

Elongator is an evolutionarily conserved protein complex [108]. hElp1-hElp4 have been identified in the literature and Elp5 and Elp6 homologues exist (C. Binda, I.

Berke and R. Collins unpublished data), however, they have yet to be fully characterized. hElp1 was first identified when it was thought to be a regulatory scaffold component that regulates IκB kinase, and was named IKAP (*I*κB *k*inase *a*ssociated *p*rotein) [109], however it was later found that hElp1 did not function in the NFκB pathway [110]. Mutations in hElp1 have been linked to the neurodegenerative disease Familial Dysautonomia (FD) [111]. The primary mutation results in a truncated protein containing only its N-terminal half. FD is mainly found in the Ashkenazi Jewish population with a carrier rate of about 1:4,000. FD patients exhibit developmental failures in the peripheral and autonomic nervous system. Our functional data involving Elp1p in the regulation of exocytosis suggest that this failure may be due to a defect in exocytosis regulation in humans possessing mutant hElp1 alleles [96].

In *S. cerevisiae* Elongator function is nonessential under normal growth conditions, however cells do display a number of phenotypes when function is lost, such as sensitivity to growth on high salt [97-99]. Also, cells that lack Elongator function exhibit sensitivity to growth on rich media with 2 mM caffeine at 40^{o}C. Caffeine is a common drug used to test for phenotypes, although the molecular target is unknown. This is a peculiar phenotype, yet a very specific phenotype. Chapter 5 will describe a phenotypic screen using the *S. cerevisiae* nonessential knockout collection to identify other proteins and potentially other pathways that also exhibit the same phenotype, which may give clues to Elongator function.

Loss of Elongator function also gives rise to resistance to the growth arrest induced by the *Kluveromyces lactis* secreted toxin, zymocin that induces G1 cell cycle arrest in limited growth nutrients [112]. Zymocin is composed of three subunits, an α subunit that has exochitinase activity, a β subunit that is required for zymocin docking and binding to chitin on the cell surface to internalize the γ toxin that can act on its

targets where it can cause cell cycle arrest. Elongator null mutants were identified, along with other genes termed *kti* genes (*k*iller *t*oxin *i*nsensitive), as genes where loss of function resulted in the ability to grow in the presence of zymocin, suggesting these proteins may be the zymocin target [112].

Functional analysis has identified three processes that Elongator function may impact: transcription elongation, exocytosis, and tRNA modification [96, 97, 113]. It was initially thought to function as a transcription elongation factor, as subunits Elp1p-Elp3p copurified with RNA PolII [97]. Biochemical analysis indicated that Elp3p may possess histone acetyltransferase activity, however it remains unclear if histone tails are its true substrate as a number of studies have questioned this function *in vivo* [96, 98, 100, 114, 115]. Conflicting evidence exists as to whether or not Elongator is a true transcription factor. It was initially identified to copurify with RNA PolII through an extensive purification protocol [97, 99]. *In vivo,* deletion of Elongator subunits gave rise to delays in growth rates after switching carbon sources, which was assessed as a defect in the onset of transcription [97, 99]. However, one of the two papers that identified Elp4p, Elp5p and Elp6p as additional holo-Elongator subunits noted that RNA PolII did not copurify with their purification of holo-Elongator [98]. This same group also further characterized the interaction between known transcription elongation factors and RNA PolII and found that Elongator subunits were the only factors that failed to bind RNA PolII [114].

A detailed genome-wide analysis of transcription initiation factor and elongation factor binding to promoters and open reading frames failed to identify Elongator on promoters or ORFs in the genome, suggesting that it did not bind DNA [115]. Furthermore, this study localized myc-tagged Elp1p, Elp2p and Elp3p to the cytoplasm and suggested that it is not an elongation factor. Work described in Chapter 4 extends this analysis using GFP-tagged versions of all six subunits and found that

each subunit localizes to the cytoplasm and does not undergo nuclear-cytoplasmic shuttling [96].

Work from our laboratory that I will discuss in Chapters 4 and 5 found Elongator negatively regulates exocytosis when it was identified as an extragenic suppressor of a temperature sensitive *sec2* allele [96]. Elp3p, a cytoplasmic resident, requires its acetyltransferase domain to negatively regulate exocytosis suggesting that the physiological target of its acetyltransferase activity is not histone tails, but a cytoplasmic protein. Other work from our laboratory indicates that Elp3p also requires its SAM-radical domain to negatively regulate secretion (I. Berke and R. Collins, unpublished results). Genetic analysis discussed in Chapter 5 suggests that the physiological effect of Elongator function on exocytosis may influence Sec4p or Sec9p function. Loss of Elongator function results in a similar genetic profile to overexpression of *SEC4* or *SEC9* suggesting this may be the area of the pathway it is regulating *in vivo*.

Elongator has also been implicated in modifying tRNAs on uridine residues at the wobble position [113]. Huang *et al* found that all six Elongator subunits, as well as *KTI11-KTI13,* are required for an early step in synthesis of 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) groups found on uridines at the wobble position in tRNA. They suggested that Kti11p – Kti13p serve as regulators of this Elongator-dependent synthesis step. Through UV-crosslinking experiments, this study found that only Elp1p and Elp3p associated with $tRNA^{GLU}$ but Elp5p did not, suggesting Elp1p-Elp3p associates with tRNA. The same group also identified that the zymocin γ toxin targets and cleaves these modified tRNAs and through this action induces growth arrest [116].

Elongator has been implicated in three functions. From data published to date, as well as unpublished data I will discuss in Chapter 5, it is unclear if these functions

are connected through a common mechanism of action or if Elongator has three independent functions and regulates multiple cellular processes. It is entirely possible that Elp3p has multiple substrates and Elongator has accessory proteins (Elp1p, Elp2p, Elp4p-Elp6p) as structural and regulatory components to recruit the different substrates. This, however, remains to be examined.

Overview

In Chapter 2 I will present work identifying and characterizing *in vivo* requirements of the COPI appendage domain function. Structural analysis by Gregory Hoffman in the Cerione Laboratory at Cornell demonstrated that the γCOP C - terminus adopts an appendage domain structurally similar to the appendages found in the AP-2 complex that recruit regulatory components to the site of vesicle budding, providing insights into COPI vesicle formation [6].

In Chapter 3 I will discuss localization studies of the Rab GTPases in *S. cerevisiae,* and particularly, where we identified the localization of the previously uncharacterized Rabs, Ypt10p and Ypt11p [40].

In Chapter 4 I will discuss data identifying the Elongator complex as a negative regulator of polarized exocytosis. A null mutant of *elp1* was identified as an extragenic suppressor of a temperature sensitive *sec2* allele. I will discuss further analysis suggesting mechanisms of how Elongator functions in this process [96].

In Chapter 5 I will present additional data further supporting Elongator's role as a negative regulator of polarized secretion. I will present an extensive genetic analysis that gives insights into the stage in the secretory pathway Elongator may act. I will also present the identification of other proteins that may function in the same pathway as Elongator to regulate membrane transport.

In Chapter 6 I will discuss localization analysis of the deacetylase enzymes in *S.cerevisiae*. Elp3p possesses an acetyltransferase activity domain and we are interested in identifying potential substrates for this activity. Identifying the deacetylase that may function in this pathway would be of great importance for substrate identification. I will show a closer analysis of Hos3p localization. Hos3p localizes to the neck region between the mother and daughter cell, a region in the cell where exocytosis takes place just prior to cytokinesis.

In Chapter 7 I will discuss experiments that describe a role for the ubiquitin-like modification, Urmylation, in the regulation of exocytosis. Similar to Elongator function, loss of *URM1* or the E1 ligase *UBA4* suppresses temperature sensitive mutations in *SEC2*. Urmylation function is not well understood and I will describe a protocol to identify the Urm1-conjugated proteins which may provide insights into how urmylation regulates secretion.

In Chapter 8 I will present a new set of RFP subcellular markers that will be beneficial to the yeast community for colocalization studies. I have cloned these genes on shuttle plasmids to further verify the NY cloning technique used in our laboratory. I verified the localization of each marker in hopes they will aid others in the lab in live microscopy studies.

In Chapter 9 I will present work where I further characterized the localization requirements of one of the RFP subcellular markers – Snq2p-RFP. This protein has an intriguing localization as it is a transmembrane protein that appears to be restricted to the mother cell for the majority of the cell cycle. Generally, plasma membrane proteins go through the secretory pathway and are deposited at the daughter cell plasma membrane, however, this protein remains excluded from this area for the majority of the cell cycle.

In Chapter 10 I will draw conclusions from my thesis work. I will discuss possible future directions that could be taken to try to gain insights into Elongator's function in regulating polarized exocytosis, as this is an area that is just beginning to be explored and could have therapeutic implications in the future to the treatment of FD.

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Chapter 2. Functional analysis of COPI appendage domains.[1]

Abstract

Coat proteins recruit cargo proteins and initiate vesicle biogenesis. Clathrin, COPI and COPII coats are the best characterized with each regulating a distinct stage of transport. Clathrin has been characterized in detail, both structurally and functionally. Appendage domains are key regulatory domains in the clathrin coat that serve to recruit accessory proteins required for vesicle formation [1-5]. Structural characterization of the γCOP subunit by Gregory Hoffman in the Cerione laboratory demonstrated that, despite limited sequence identity, the γCOP C-terminus adopts a similar tertiary structure to the AP2 appendage domains [6]. Functional analysis of the γCOP homolog in yeast, *SEC21*, revealed that the C-terminus is required for function. Furthermore, sequence and functional analysis suggest that βCOP and its homolog *SEC26* has a putative appendage domain at the C-terminus. The functional analysis presented here suggest that the COPI coat may assemble and function similarly to the clathrin coat to regulate vesicle formation for retrograde transport from the Golgi.

Introduction

Eukaryotic cells transport most of their cellular material between organelles in membrane-bound coated vesicles. Three coats have been characterized in detail, COPI, COPII and clathrin, where each coat regulates a distinct stage of transport [7-12]. The clathrin coat is the best understood coat, as it has been functionally and

[1] Portions of this work were published in Hoffman, GR, *et al.* Molecular Cell (2003) 12, *3*, pg. 615-625.

structurally characterized. The clathrin coat regulates endocytic transport from the plasma membrane and *trans* Golgi to endosome transport [12].

Structurally, the clathrin coat consists of the adaptin complex and three-legged clathrin triskelions. The heterotetrameric adaptin complex recruits clathrin to sites of vesicle formation. Polymerization of triskelions through contacts between leg domains leads to coat formation to generate membrane curvature to initiate vesicle budding. Electron micrographs revealed that the adaptin complex adopts a characteristic structure consisting of a trunk domain with two appendage domains emanating from it [13]. The appendage domains are located at the C-terminus end of the two large subunits and connect to the trunk domain by a flexible linker. The appendage domains serve as recruitment hubs for accessory factors to aid in vesicle biogenesis [4, 5].

The mechanism for COPI and COPII coat assembly and function is less understood. COPI coats form on Golgi-derived vesicles and consist of seven conserved subunits (α, β, β', δ, γ, ε, and ζ). GTP-bound Arf1 plays a role in the recruitment of COPI subunits to sites of vesicle formation. The COPI complex can be dissociated into two discrete subcomplexes, B-COPI and F-COPI [14]. F-COPI is composed of β, δ, γ, and ζ while B-COPI consists of the three remaining subunits (Figure 2.1). The F-COPI subunits exhibit low sequence identity to subunits in the adaptin complexes. For example, β- and γCOP share significant homology with α- and βAP2 subunits at the N-termini that form the trunk domains of each protein. Biochemical and sequence analysis suggest that COPI may adopt a similar overall architecture where F-COPI is analogous to the adaptin complex and B-COPI is analogous to clathrin [8, 11].

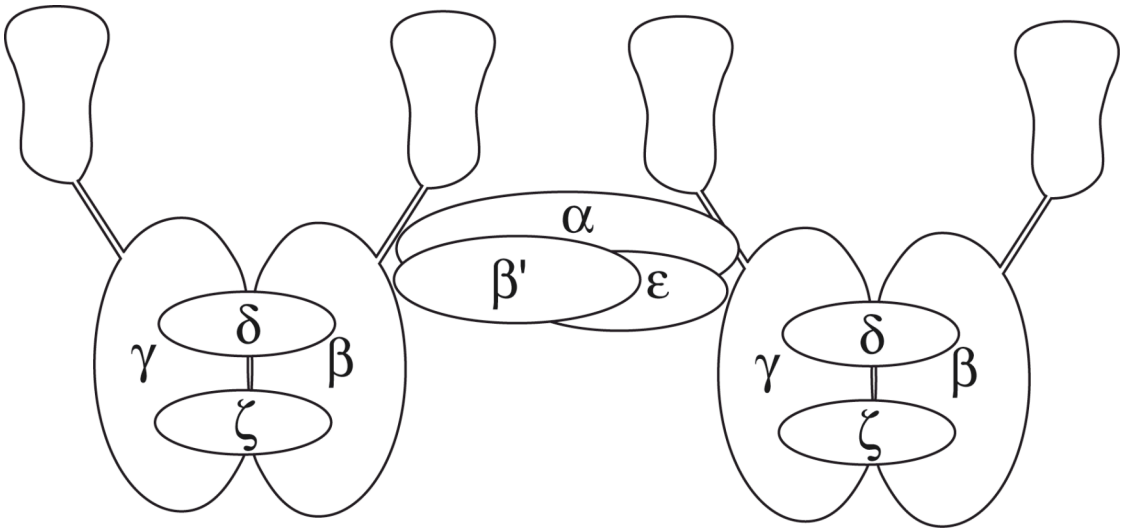

Figure 2.1. Model for COPI coat formation. A schematic representation of COPI subunit interactions where α, β, β', γ, ζ and δ are COPI subunits. Adapted from [6].

Structural characterization of the γCOP C-terminus revealed striking structural homology with the appendage domains of α and β subunits of AP2 adaptins despite low primary sequence homology (Figure 2.2) [6]. All seven COPI subuits are conserved throughout evolution with homologues in *S.cerevisae,* including the γCOP homologue, Sec21p, and the βCOP homologue, Sec26p, which allowed for functional characterization of the γCOP/Sec21p C-terminal appendage domain. Functional analysis of Sec21p revealed a requirement of its C-terminal appendage for function [6]. Further, the C-terminal appendage function is evolutionarily conserved as a chimeric *SEC21* containing the yeast N-terminus and a mammalian C-terminus can complement at *SEC21* deletion.

Additional functional analysis on the Sec21p appendage domain will be presented here, along with the identification of a putative appendage domain on

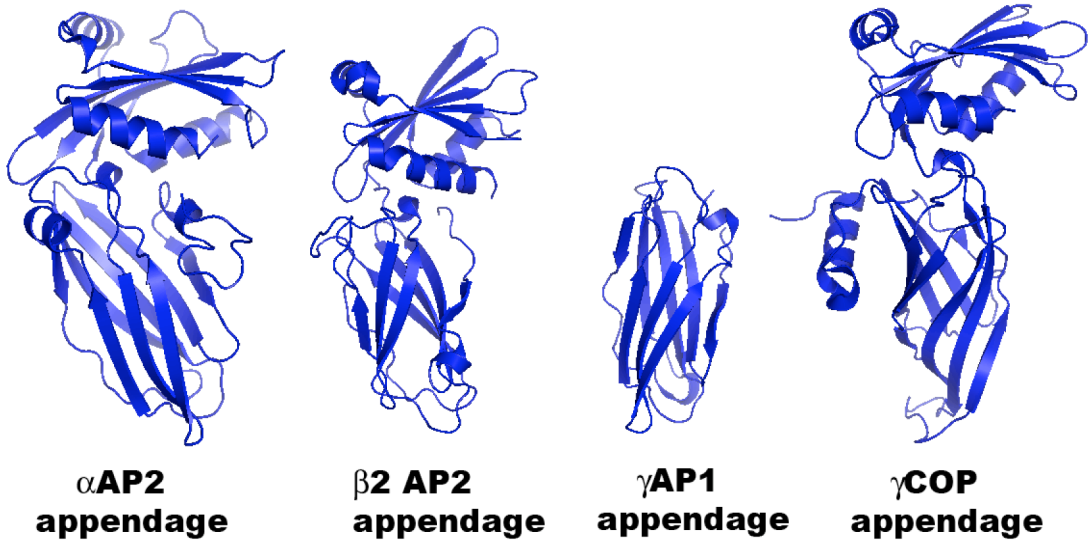

Figure 2.2. Three-dimensional structures of AP2 and COPI appendage domains. Appendage domain structure from AP2 and COPI subunits illustrating the structural conservation between the proteins. 1QTP (αAP2), 1E42 (βAP2), 1GYU (γAP1) and 1PZD (γCOP) structures were used and figures were created using MacPyMOL [2, 3, 6, 15].

Sec26p, the βCOP homologue, that, similar to Sec21p, is required for function. Functional data suggests that the Sec21p and Sec26p appendage domains function in close concert. Our current model places COPI appendage function in an analogous fashion to the adaptin appendage domains where they may act as hubs to recruit factors that influence vesicle biogenesis.

Materials and methods

Functionality studies of the Sec21p and Sec26p appendage domains were carried out in a *SEC21 SEC26* tester strain (RCY2590 *MATα his3Δ0 leu2Δ0 ura3Δ0 lys2Δ0 SEC21ΔHIS3 SEC26ΔKAN*^{R} [pRS316 *SEC21 SEC26*]). Mutant and wild type constructs were generated using standard molecular biology procedures. The tester strain was transformed with *LEU2*-containing plasmids for *SEC21* and *LYS2*-containing plasmids for *SEC26*. Transformants were plated on media containing

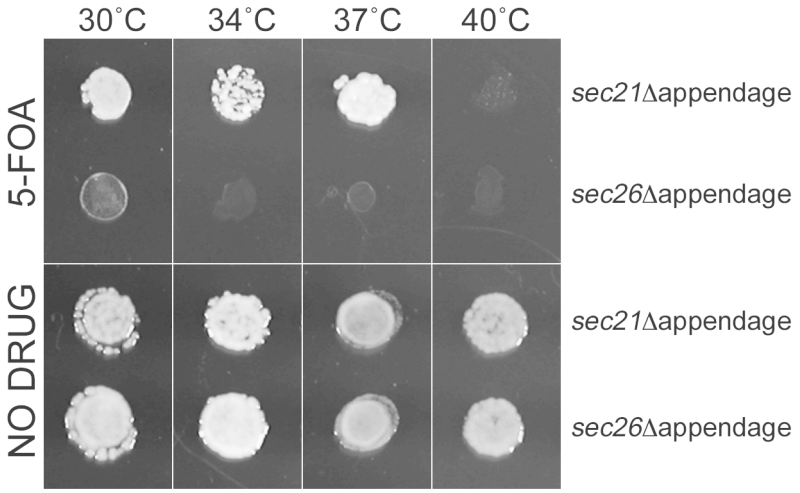

Figure 2.3. Functional requirement for *SEC21* and *SEC26* appendage domains. Growth of *sec21* and *sec26* appendage domain mutants was tested in the *SEC21 SEC26* tester strain. RCY2590 cells were simultaneously transformed with pRS315 *sec21* or pRS317 *sec26* plasmids. Transformants were spotted on 5-FOA plates to eliminate the pRS316 *SEC21* and *SEC26* plasmid to test the functionality of each mutant at the indicated temperatures.

2mg/ml 5-FOA to induce loss of the wild type genes (maintained on *URA3*-containing plasmids) and viability was assessed at various temperatures.

Constructs were generated using the overlap PCR cloning method. Restriction digestion sites were engineered at the sites of the point mutations for ease in isolation. Constructs containing the appropriate insert were determined using restriction enzyme digestion followed by DNA sequencing.

Results

SEC21 and *SEC26* appendage domains are critical for function

Structural characterization of the γCOP C-terminus indicated a domain that structurally resembles the AP2 adaptins appendage domains [6]. Initial functional analysis using the *S.cerevisiae* γCOP homologue, *SEC21*, indicated that this domain is required for function and evolutionarily conserved. A yeast-mammalian chimera with

the yeast N-terminus and mammalian C-terminus can compliment a null *SEC21* allele using the 5-FOA plasmid shuffle assay [6]. Primary sequence analysis and secondary structure predictions suggest that βCOP contains a putative appendage domain. Therefore, we wished to further investigate the COPI appendage domains and their *in vivo* function.

SEC21 and *SEC26* both require their C-terminal appendage domains for function (Figure 2.3). Truncation of *SEC21* (residues 1-676) or *SEC26* (residues 1-593), generating *sec21Δappendage* or *sec26Δappendage,* creates a nonfunctional protein that cannot serve as the sole copy of each protein, indicating these domains are critical for function. *sec26Δappendage* causes a more drastic phenotype than *sec21Δappendage*, as *sec26Δappendage* is required for growth at all temperatures tested, whereas *sec21Δappendage* is temperature sensitive only at high temperatures. We wished to further investigate the role of the appendage domains through additional mutational analysis.

The *SEC26* platform subdomain is required for function

Structural analysis of γCOP appendage domain demonstrated that it adopts a similar tertiary structure to the α and β AP2 adaptin complex appendage domains [6]. The appendage domain consists of two domains, the immunoglobulin-like subdomain and the platform subdomain (Figure 2.4). The C-termini of the AP2 appendage domains are called platform domains as they serve as a binding platform for a number of proteins with roles in mediating clathrin-coated vesicle formation [1, 3]. Our initial mutational analysis revealed that loss of the *SEC26* appendage domain displays a stronger phenotype than the *SEC21* appendage and therefore we carried out further truncation analysis to investigate the *SEC26* appendage domain. We reasoned that the

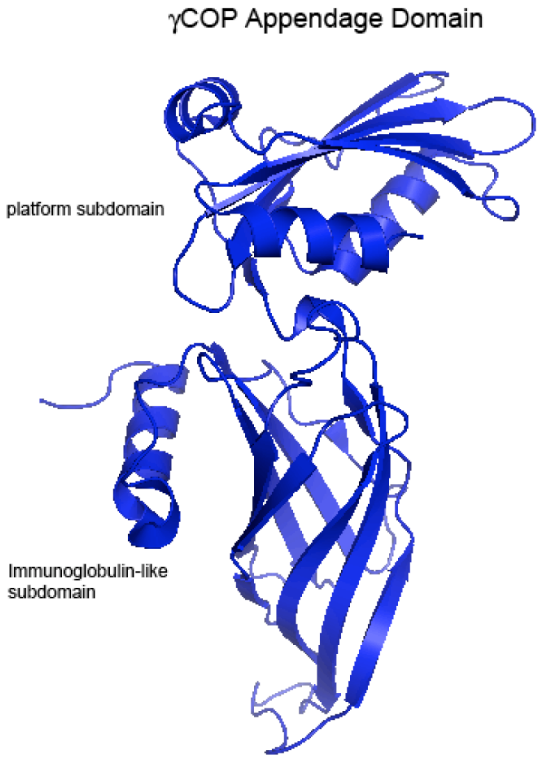

Figure 2.4. Platform and immunoglobulin domains of the γCOP appendage. The γCOP appendage (1PZD) is composed of two subdomains: the immunoglobulin (*bottom*) and platform (*top*) subdomains. Adapted from [6].

difference in phenotypes might suggest the Sec26p appendage domain is more critical than the Sec21p appendage for vesicle biogenesis.

The *SEC26* platform subdomain was determined as residues 843-973 using primary sequence analysis and secondary structure predictions. Truncation of the *SEC26* platform domain (*sec26Δplatform*) also results in a nonfunctional protein, similarly to the *sec26Δappendage* mutant, indicating that the putative platform domain is required for *SEC26* function (Figure 2.5). This analysis suggests that the *SEC26* platform domain may be functionally analogous to the AP2 platform domains to recruit factors to regulate COPI vesicle formation. Further biochemical

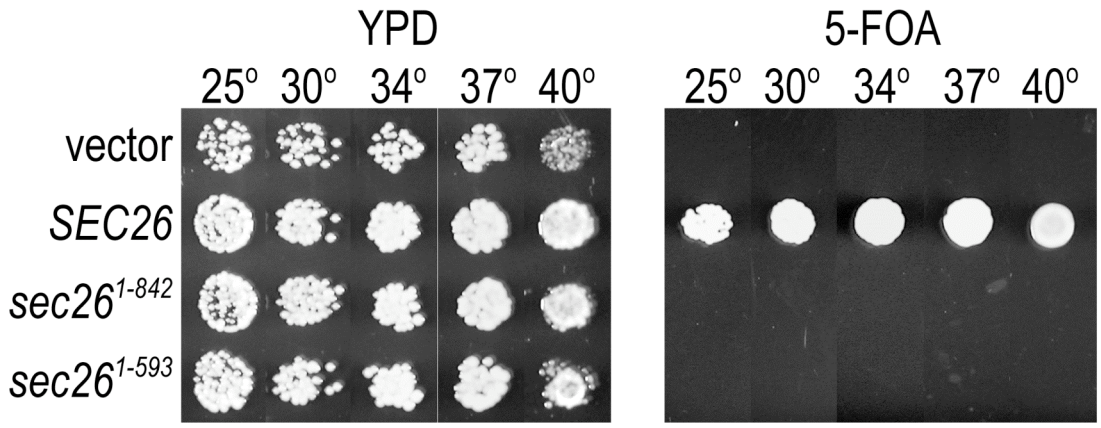

Figure 2.5. Mutational analysis of *sec26* putative appendage subdomains. pRS317 *sec26* plasmids were transformed into *SEC26* tester strain. Transformants were spotted on 5-FOA plates to eliminate the pRS316 *SEC26* plasmid to test the functionality of each mutant at the indicated temperatures. $sec26^{1-842}$ is a platform subdomain truncation and $sec26^{1-593}$ is an appendage domain truncation. Figure courtesy of Carol DeRegis.

characterization of this domain will be required to demonstrate a role for the *SEC26* platform domain in recruitment.

Mutation analysis of the conserved F/W motif in *SEC21* and *SEC26*

Truncation of the *SEC21* and *SEC26* C-termini indicated an essential functional domain that may be analogous to the αAP2 and βAP appendage domains. An important conserved feature of the AP2 platform domains is a pair of aromatic residues present in γCOP (F772 and W776 in γCOP), termed the F/W motif [6]. A conserved F/W motif is apparent in βCOP when aligned with γCOP, a feature that can be identified in most γCOP, βCOP, αAP2 and βAP2 homologues, suggesting this motif plays an important role in function of all appendage domains. In terms of αAP2

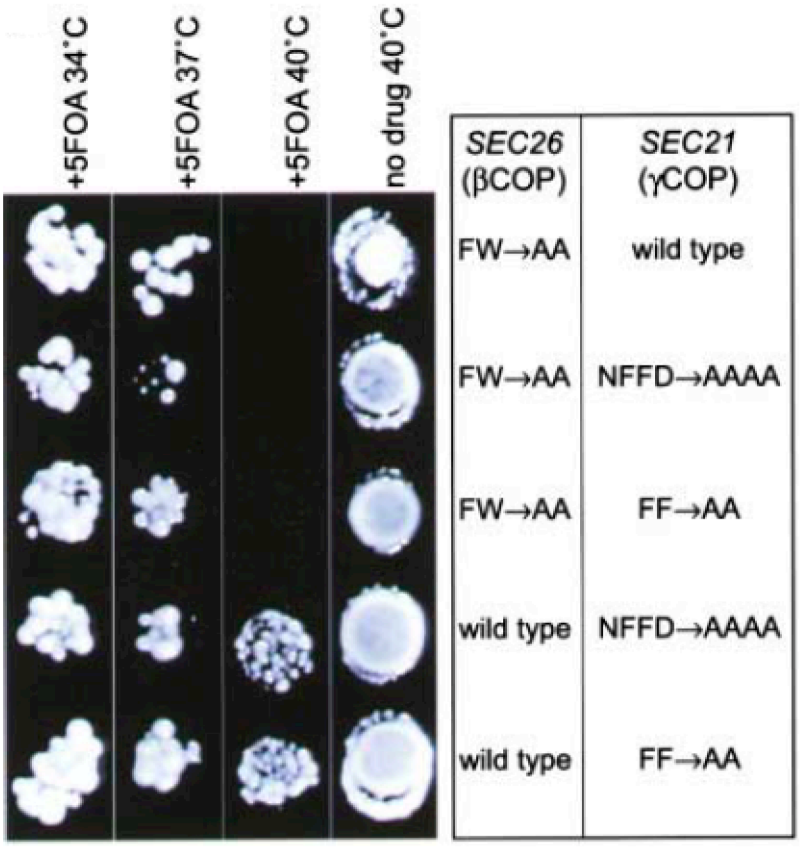

Figure 2.6. *SEC26* F/W motif is required for function. Growth of *sec21* and *sec26* point mutants was tested in the *SEC21 SEC26* tester strain. RCY2590 cells were simultaneously transformed with pRS315 *sec21* or pRS317 *sec26* plasmids. Transformants were spotted on 5-FOA plates to eliminate the pRS316 *SEC21* and *SEC26* plasmid to test the functionality of each mutant at the indicated temperatures.

and βAP2, the F/W motif, along with the surrounding conserved aromatic residues, influence appendage domain interactions with binding partners [1-3].

SEC26 contains the F/W motif while the tryptophan in *SEC21* has a conservative phenylalanine substitution [6]. Mutation of the F/W motif in Sec26p (*sec26*$^{F856A, W860}$) gives rise to temperature sensitivity, indicating a requirement for these residues for function (Figure 2.7). In contrast, mutation of the corresponding region in Sec21p (*sec21*$^{F832A, F836A}$) has no apparent effect on cell growth, as does

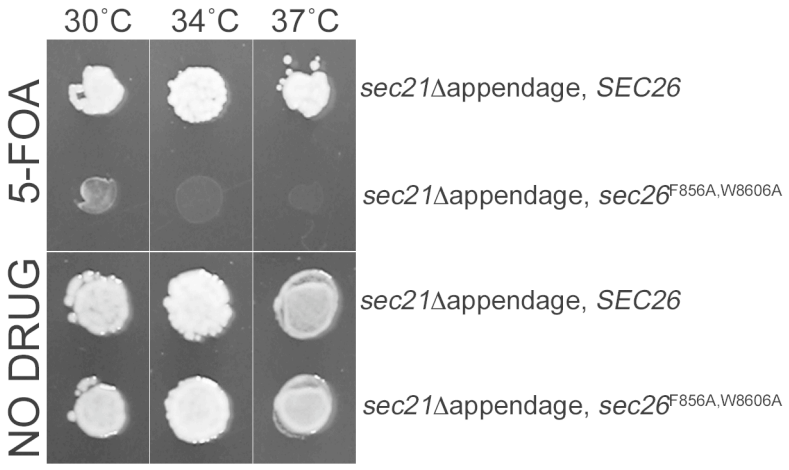

Figure 2.7. Synthetic lethal analysis of *sec21* and *sec26* mutants. Growth of *sec21* and *sec26* mutants was tested in the *SEC21 SEC26* tester strain. RCY2590 cells were simultaneously transformed with pRS315 *sec21* or pRS317 *sec26* plasmids. Transformants were spotted on 5-FOA plates to eliminate the pRS316 *SEC21* and *SEC26* plasmid to test the functionality of each mutant at the indicated temperatures.

mutation of the flanking conserved amino acids ($sec21^{N831A,\ F832A,\ F836A,\ D836A}$) (Figure 2.6). The lack of requirement for the Sec21p F/W motif may suggest that the Sec26p/βCOP appendage domain can functionally substitute for loss of phenylalanine 832 and 836 in Sec21p. This is supported from data showing that $sec26^{F856A,\ W860}$ combined with *sec21Δappendage* causes a lethal phenotype. The $sec26^{F856A,\ W860}$ mutant does not have a synthetic effect on cell growth in combination with a deletion the *sec26* and *sec21* genetic interactions are specific. Synthetic lethality is frequently observed between gene products that act in the same pathway and supports the idea that the appendage domains of *SEC21* and *SEC26* act in close concert.

Discussion

The data presented here implicate the C-terminal domains of COPI subunits *SEC21* and *SEC26* as regions of functional importance. Truncation analysis found that

the *SEC21* and *SEC26* appendage domains are required for function. Further, the platform domain and the F/W motif verified a functionally significant domain in Sec26p, providing *in vivo* connections between the appendage domains in the COPI and clathrin coats. The functional data, taken with the structural characterization of the γCOP C-terminus, indicates that these domains may function in COPI vesicle biogenesis similar to the AP2 appendage domains to recruit factors to facilitate vesicle formation and suggests an underlying conservation in COPI and clathrin coat assembly [6].

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Chapter 3. Analysis of Rab GTPase Localization using GFP-tagged proteins Identifies the Localization of the Uncharacterized *S.cerevisiae* Rabs Ypt10p and Ypt11p[2]

Abstract

Rab GTPases are key regulators of vesicle and organelle transport. Rab proteins are peripheral membrane proteins that insert into the membrane via prenyl modifications. Proper membrane targeting is essential for function. The analysis presented here utilizes GFP-tagged Rabs in *S.cerevisiae* to characterize their localization. Functional analysis demonstrates that N-terminally GFP tagged Rab proteins are functional proteins. The analysis presented here uses GFP-tagged Rab GTPases to characterize the localization as the sole copy in cells. This strategy is used to determine the localization of the two uncharacterized Rabs: Ypt10p and Ypt11p. GFP-Ypt10p localizes to the endoplasmic reticulum and GFP-Ypt11p localizes as a punctate pattern in the endocytic pathway.

Introduction

A key feature of all eukaryotic cells is their ability to compartmentalize proteins and lipids into membrane-bound organelles. Regulating the flow of macromolecules between compartments is critical for cell growth and maintaining cell homeostasis. Rab GTPases are essential for cell growth and are one of the major regulators of the endomembrane system. *S.cerevisiae* cells have 11 Rab proteins while the human genome encodes for around 60 [1, 2].

[2] Portions of this work were published in Buvelot Frei, *et al.* Molecular and Cellular Biology (2006) 26, *19*, pg. 7299-7317.

Rab GTPases can be found in two environments: cytosolic or membrane-bound. Rabs are inserted into membranes, on the cytosolic leaflet of the bilayer, through C-terminal prenylation moieties and reside in the cytosol through heterodimerization with Rab GDI (*G*DP *D*issociation *I*nhibitor). Each Rab has a distinct steady-state localization, thought to reflect the stage of membrane transport that is regulated. The distinct events required for Rab localization remain unclear. Is localization achieved through recruiting the Rab from its cytosolic association with Rab GDI onto a distinct membrane or is the Rab already present on membranes and recruited to its proper cellular location through other Rab-interacting factors? Regardless, proper localization of Rab proteins is critical for function. Our laboratory recently described an analysis of Rab localization and the conservation of the factors necessary for proper Rab localization [3]. In this analysis, the localization was characterized for each Rab protein in *S.cerevisiae* using GFP-tagged Rab proteins. Further investigation found that expression of yeast Rabs in mammalian cells led to a similar localization, indicating that the localization factors are conserved across species.

I will discuss localization analysis of six Rab proteins in yeast, two of which previously had an unknown localization. Ypt1p and Sec4p are two well-characterized Rab GTPases that regulate Golgi and post-Golgi transport, respectively [4-6]. Ypt31p and Ypt32p are two redundant late-Golgi Rabs that we found have slightly different localizations at various stages in the cell cycle [7]. Ypt11p localizes to the endoplasmic reticulum and work from other authors of the study found its function influences ER inheritance during cell division [3]. Ypt10p is a an uncharacterized Rab that we found is involved in endocytic processes and localizes to regions on the vacuole [3].

Table 3.1. Yeast strains used in this study.

Strain number	Genotype
RCY1887	*MAT ura3-52 leu2-3,112 his3Δ200 YPT1ΔHIS3* [pRS315 *GFP-YPT1* pRC2100B]
RCY2197	*MATα/**a** ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 lys2Δ0/LYS3 met15Δ0/MET15 ypt31Δ::KAN^R/ypt31Δ::KAN^R*
RCY2160	*MATα/**a** ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 lys2Δ0/LYS3 met15Δ0/MET15 ypt32Δ::KAN^R/ypt32Δ::KAN^R*
RCY2194	*MATα/**a** ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 lys2Δ0/LYS3 met15Δ0/MET15 ypt11Δ::KAN^R/ypt11Δ::KAN^R*
RCY2205	*MATα/**a** ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 lys2Δ0/LYS3 met15Δ0/MET15 ypt10Δ::KAN^R/ypt10Δ::KAN^R*
RCY239	*MATα ura3-52 leu2-3,112*
RCY3803	*MAT ura3-52 leu2-3,112 his3Δ200 SEC4ΔHIS3* [pRS315 *GFP-SEC4^{F45A}*]
RCY3813	*MAT ura3-52 leu2-3,112 his3Δ200 SEC4ΔHIS3* [pRS315 *GFP-SEC4^{F49A}*]

Materials and methods

Yeast strains, plasmids and media

Yeast strains used in this study are presented in Table 3.1 and the plasmids are in Table 3.2. YPD (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), and SD (0.1% yeast nitrogen base, 2% D-glucose, plus required nutrients) media was used. Strains were generated for localization analysis using standard techniques and selected for on minimal media. Positive transformants were grown overnight in minimal media to log phase at room temperature prior to microscopy.

Table 3.2. Plasmids used in this study.

Plasmid number	Plasmid
RCB653	*GFP-SEC4* pRS315
RCB2606	*Gal4 binding domain-RFP* pRS316
RCB647	*GFP-YPT31* pRS315
RCB630	*GFP-YPT31* pRS315
RCB3268B	*GFP-YPT10* P_{CUP1} pRS316
RCB3267B	*GFP-YPT10* P_{YPT10} pRS316
RCB3269A	*YPT10* P_{CUP1} pRS316
RCB3335A	*GFP-YPT11* P_{YPT11} pRS315
RCB2100B	*GFP-YPT1* pRS315

YPT11 and *YPT10* Construct Creation:

The *YPT11* ORF and promoter were amplified using PCR from genomic DNA and eGFP was amplified using PCR from a plasmid template containing eGFP. *GFP-YPT10* was created with endogenous promoter using overlap PCR and cloned into pRS316. *YPT10* P_{cup1} and *GFP-YPT10* P_{cup1} constructs were created using overlap PCR and cloned into pRS316. ORF expression is controlled with the *CUP1-1/*YHR053C promoter, consisting of 330 bases from the start of *CUP1*, and endogenous *YPT10* terminator. The *YPT10* ORF and *CUP1-1* promoter were amplified using PCR from genomic DNA and eGFP was amplified using PCR from a plasmid template containing eGFP. *GFP-YPT10* was created with endogenous promoter using overlap PCR and cloned into pRS316. *YPT10 ORF* and *YPT10* promoter, consisting of 199 bases from the start of *YPT10*, were amplified using PCR from genomic DNA and eGFP was amplified using PCR from a plasmid template containing eGFP. Restriction enzyme digestion was used to determine that the constructs generated were correct. Functional analysis, through demonstrating that overexpression of GFP-tagged *YPT10* using the copper inducible promoter, verified that the GFP tag does not interfere with causing cell lethality when overexpressed,

which is a method of determining functionality. The localization of GFP-Ypt10p P_{CUP1} when expressed at basal levels was compared with the localization of GFP-Ypt10p P_{YPT10} as the final confirmation that GFP-Ypt10p is functional.

Fluorescence Microscopy:

Live cells were analyzed with a Nikon Eclipse E600 microscope, 100X (1.4 NA) lens, 1X optavar (0.08 μm/pixel), and imaged using a Sensicam EM High Performance camera (The Cook Corporation). Fluorescent images were collected as a z-series with a 0.2 μm z step size. Differential interference contrast (DIC) images were taken at one z plane. Images were captured with IP Lab 3.6.5 software and Autodeblur and Autovisualize 9.1 software was used for blind deconvolution with 30 iterations.

FM4-64 endpoint and GFP-Ypt10p colocalization studies were done incubating the cells in 2.5 mM FM4-64 in media for 15 minutes; cells were washed twice and chased for 45 minutes at 30^{o}C in fresh media. Deconvolution microscopy was done on the live cells in the FITC and TxRed channels following the protocol above.

Results

Localization analysis of the well-characterized Rab proteins: Ypt1p and Sec4p

Ypt1p was one of the first characterized Rab proteins in yeast and is an essential protein that regulates Golgi transport [6]. *YPT1* is conserved throughout evolution, where *RAB1* encodes its human homologue [8, 9]. Analysis by Monica Calero in the laboratory found that a GFP-tagged version of *YPT1* can function as the sole copy in cells through determining that GFP-Ypt1p can compliment *YPT1Δ* and allow for cell viability [3]. GFP-Ypt1p has a Golgi localization in live cells when

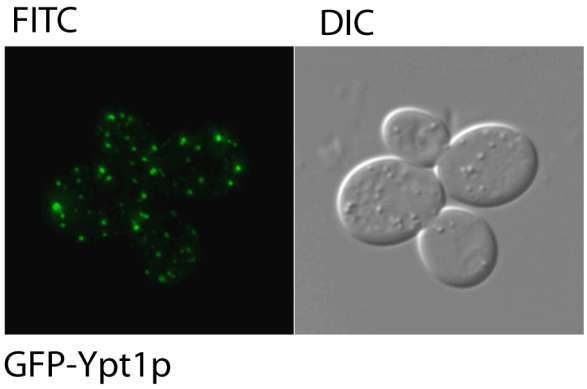


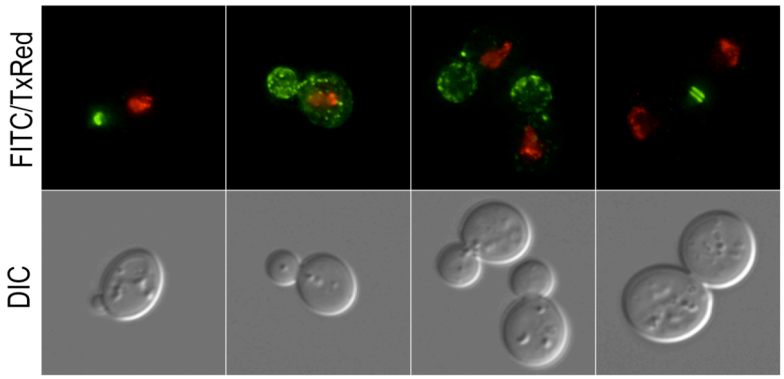
Figure 3.1. Localization of GFP-Ypt1p. GFP-Ypt1p was expressed in yeast cells as the only copy and live cells were viewed by fluorescence microscopy. The fluorescence image was taken as a z-series and the resulting deconvolved maximum projection and corresponding differential interference contrast (DIC) image is shown.

expressed as the sole copy, as displayed in Figure 3.1. The protein has a punctate localization that is evenly distributed through the mother and daughter cells.

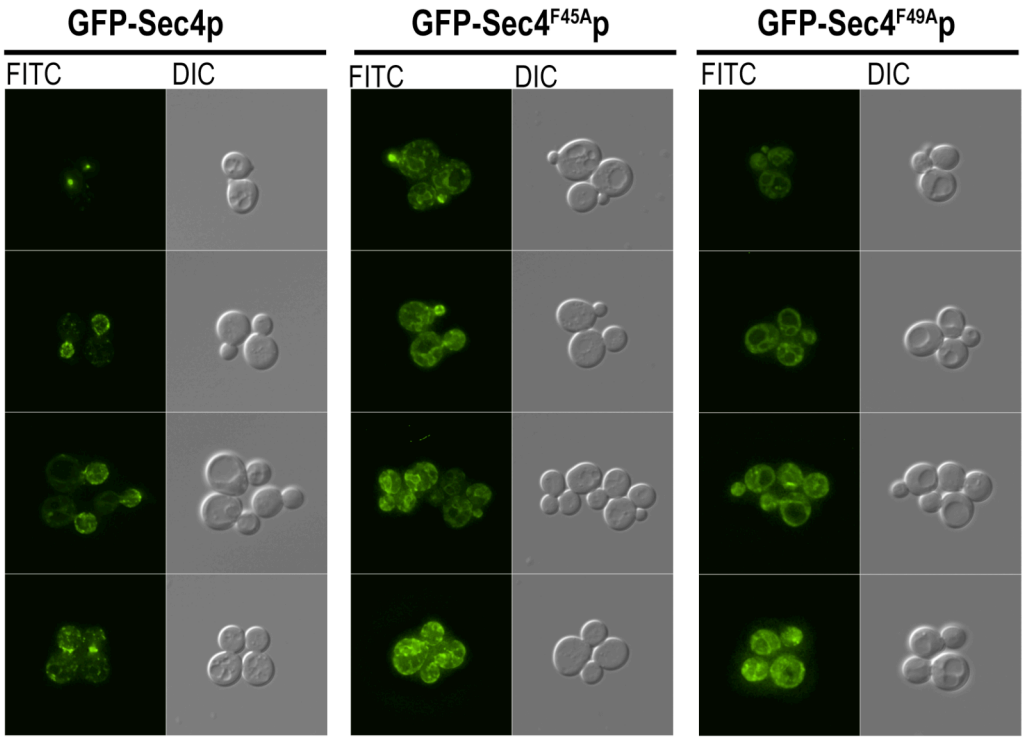
Sec4p regulates exocytic events, is found on secretory vesicles and is often used as a marker for sites of exocytosis [4, 5]. Its human counterparts include Rab3a and Rab8 [8, 9]. Similar to GFP-Ypt1p, GFP-Sec4p can function as the sole copy of *SEC4*, which encodes an essential protein. GFP-Sec4p displays a cell-cycle dependent localization, as depicted in Figure 3.2a. In this analysis, the state of the cell cycle is monitored through daughter cell size and with an RFP nuclear marker. Early in the cell cycle, GFP-Sec4p localizes to the emerging daughter cell. As cell growth continues, the daughter cell gets larger and GFP-Sec4p is enriched at the daughter cell plasma membrane periphery. Following nuclear migration and division but prior to cytokinesis, GFP-Sec4p is reoriented towards the neck region between the dividing mother and daughter cells.

Figure 3.2. Sec4p cell cycle-dependent localization. A) GFP-Sec4p was expressed in yeast cells as the only copy and live cells were viewed by fluorescence microscopy. GFP-Sec4p localization was analyzed at various stages of the yeast cell cycle, using the nucleus (Gal4-RFP) and bud size as a measure of the stage of the cell cycle. The corresponding differential interference contrast (DIC) image is also shown. The FITC and TxRed channel images display the maximum projection from a deconvolved z-series. B) Localization analysis of GFP-tagged Sec4p mutants in live cells at room temperature. FITC images were taken as a z-series and a resulting deconvolved slice is shown from the center of the cell with the corresponding DIC image. Four representative images are shown for each mutant.

A) **GFP-Sec4p, red nucleus**



B)



Maria Nussbaum in the laboratory designed an algorithm that utilized multiple sequence alignments to identify critical residues required for Sec4p function [10]. Two phenylalanine residues lying in loop 2, F45 and F49, were identified as potential residues required for Sec4p function. She mutated each residue to an alanine to analyze the effect of each residue on Sec4p localization.

We found that mutation of either residue caused GFP-Sec4p to mislocalize. GFP-Sec4^{F45A}p appears to be nonspecifically distributed throughout the endomembrane system and more cytosolic compared to wildtype (Figure 3.2b). GFP-Sec4^{F49A}p has a similar mislocalization but is slightly more cytosolic than the F45A mutant. It is important to note that GFP-Sec4^{F45A}p is concentrated in small budded cells at sites of exocytosis in addition to localizing to, what appears to be, other membranes. This analysis found that Sec4p requires F45 and F49 to maintain a proper localization at sites of exocytosis.

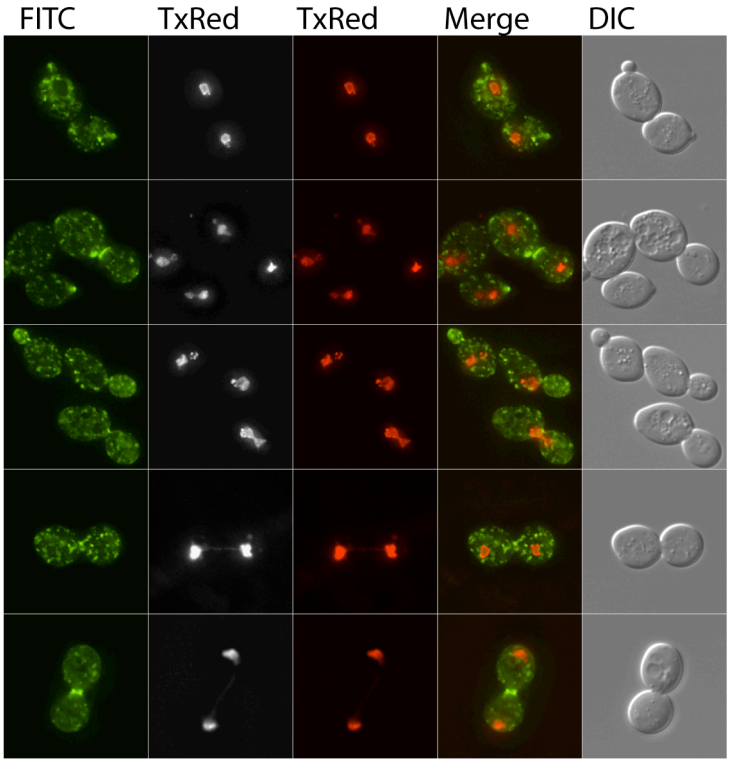
Localization analysis of the Golgi Rabs Ypt31p and Ypt32p

Ypt31p and Ypt32p are redundant Rab proteins that appear to function within the Golgi and in post-Golgi events and is homologous to Rab11 [11]. Each protein is non-essential, however, loss of both functions causes lethality. We independently expressed GFP-Ypt31p and GFP-Ypt32p as the only cellular copy and monitored the localization throughout the cell cycle (Figure 3.3a,b). Similar to GFP-Sec4p, GFP-Ypt31p and –Ypt32p have a cell cycle dependent localization.

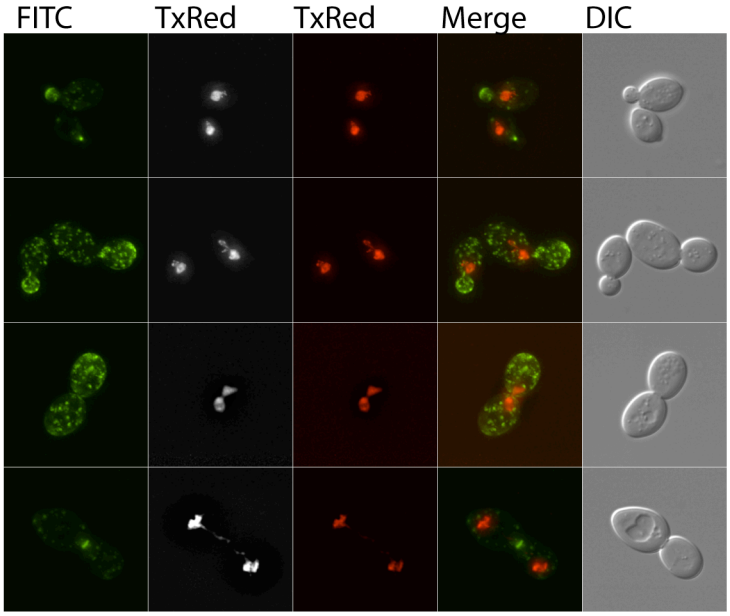
In small budded cells, both proteins have similar but not identical distributions. GFP-Ypt32p showed distinctive polarized bud tip staining as well as some scattered Golgi-like puncta. The polarized distribution of GFP-Ypt31p was less pronounced and was visible as a clustering of Golgi-like puncta towards the bud tip. In dividing cells, we observed different distributions as well. GFP-Ypt31p localized

Figure 3.3. Localization of GFP-Ypt31p and GFP-Ypt32p. A) GFP-Ypt31p was expressed in yeast cells as the only copy, in *ypt31ΔKAN/ypt31ΔKAN* diploids, and live cells were viewed by fluorescence microscopy. GFP-Ypt31p localization was analyzed at various stages of the yeast cell cycle, using the nucleus (Gal4-RFP) as a measure of the stage of the cell cycle. The corresponding differential interference contrast (DIC) image is also shown. The FITC channel image displays the maximum projection from a deconvolved z-series. The TxRed channel image displays the maximum projection in the top two series and a deconvolved slice selected from the z stack for the bottow two series of images. The overlay uses the deconvolved maximum projection of each channel. B) GFP-Ypt32p was expressed in yeast cells as the only copy, in *ypt32ΔKAN/ypt32ΔKAN* diploids and live cells were viewed by fluorescence microscopy. GFP-Ypt32p localization was analyzed at various stages of the yeast cell cycle, using the nucleus (Gal4-RFP) as a measure of the stage of the cell cycle. Differential interference contrast (DIC) images were taken in one z plane. GFP-Ypt32p and Gal4-RFP fluorescence of the same cells were analyzed using between 25-30 z-stacks. The FITC channel image displays the maximum projection from a deconvolved z-series. The TxRed channel image displays the maximum projection in the top three series and a deconvolved slice from the cell center for the bottow two series of images. The overlay uses the deconvolved maximum projection of each channel.

GFP-Ypt31p in *ypt31*Δ/*ypt31*Δ



GFP-Ypt32p in *ypt32*Δ/*ypt32*Δ



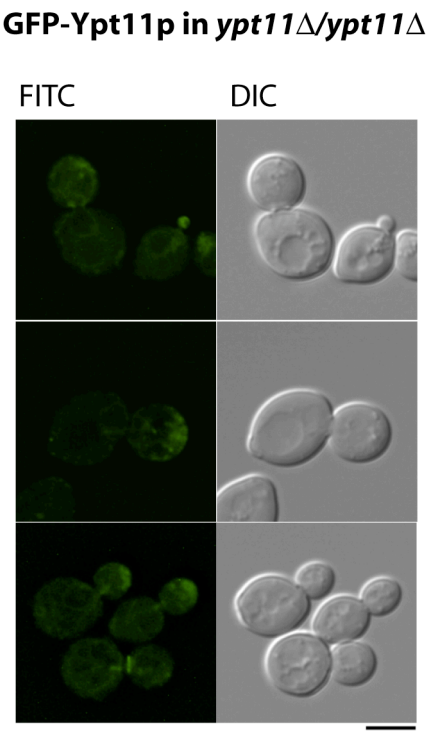


Figure 3.4. GFP-Ypt11p localizes to the endoplasmic reticulum. GFP-Ypt11p was expressed in yeast cells as the only copy, in *ypt11ΔKAN/ypt11ΔKAN* diploids, and live cells were viewed by fluorescence microscopy. The FITC channel image displays a slice from a deconvolved z-series of the center of the cell from three separate images. The corresponding differential interference contrast (DIC) image is shown. Size bar is 5μm.

predominantly to the neck of dividing cells, a discrete single ring that probably represents the area of cytokinesis. This distribution is similar to Sec4p and Sec3p localization, two proteins involved in polarized exocytosis [4, 12]. GFP-Ypt32p displays scattered Golgi-like puncta, which appear to be evenly distributed throughout the two dividing cells with some enrichment in the neck region during cytokinesis. Similarly, when expressed in HeLa cells, each protein had slight differences in localization suggesting that Ypt31p and Ypt32p, although redundant, may have slightly different functions *in vivo* [3].

Ypt11p localizes to the ER and functions in ER inheritance

Prior to our analysis, the function and localization of Ypt11p were unclear, however, it was known to interact with the class V myosin Myo2p and required for the retention of newly inherited mitochondria [13, 14]. Bioinformatic two dimensional principal component analyses indicated that Ypt11p clustered with biosynthetic/exocytic Rabs, in the "Rab 8" group [3, 15]. When expressed in cells as a GFP-fusion protein, GFP-Ypt11p localizes to a reticular-like structure surrounding the nucleus (Figure 3.4) [3]. GFP-Ypt11p also displays a partially polarized localization towards the daughter cell, suggestive of the peripheral endoplasmic reticulum (ER). Further analysis by other authors of the manuscript found functional significance in Ypt11p localization [3]. Cells lacking *ypt11* display defects in ER inheritance, similar to that of a *myo4Δ* cell strain [3, 16]. In addition, a double mutant strain lacking both *ypt11* and *myo4* has severely pronounced defects using two markers for the ER. This analysis suggests that the uncharacterized Rab GTPase Ypt11p localizes to the ER and functions in regulating ER inheritance during cell division.

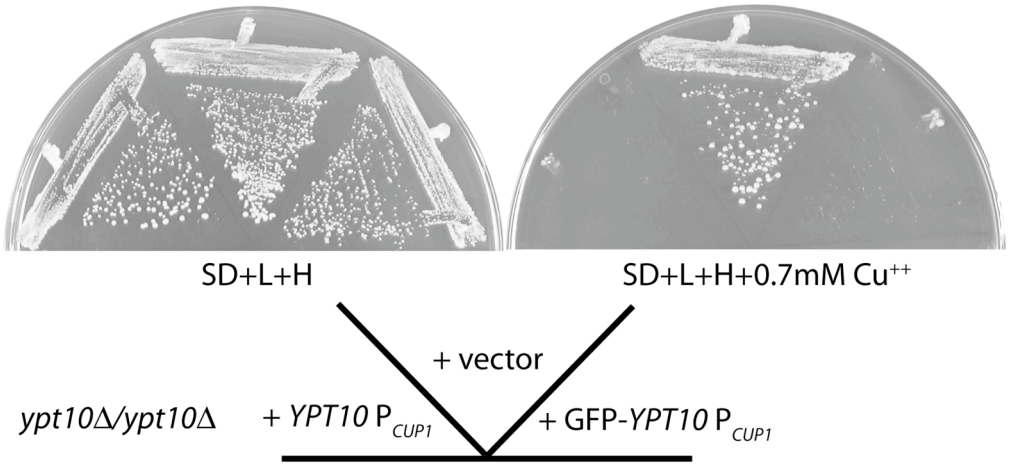
Ypt10p is an endocytic Rab localizing to the regions on the vacuole

Ypt10p is an uncharacterized Rab that is growth inhibitory when overexpressed [17]. Similarly, GFP-Ypt10p is also growth inhibitory when overexpressed, suggesting that GFP-tagging Ypt10p does not disrupt function (Figure 5a). When expressed in cells, GFP-Ypt10p localizes to membranous structures, some of which were often in the shape of small puncta, often closely associated with the vacuolar membrane (Figure 3.5b). In some cases, faint labeling of the plasma

Figure 3.5. GFP-Ypt10p localizes to regions on the vacuole. A) *YPT10* and *GFP-YPT10* constructs were generated with the ORF expression controlled using the copper promoter. *YPT10* P_{cup1}, *GFP-YPT10* P_{cup1}, and vector only were transformed into *ypt10ΔKAN/ypt10ΔKAN* diploid yeast and selected for on selective media. Transformants were streaked on SD+leu+his and SD+leu+his+0.7mM Cu^{++} to test for functionality of GFP tagged *YPT10*. Plates were incubated at 25°C. B) GFP-Ypt10p localization was determined in live cells serving as the sole copy in *ypt10ΔKAN/ypt10ΔKAN* diploid cells, under the control of the endogenous promoter (*left*) or copper promoter (*right*). Differential interference contrast (DIC) images were taken in one z plane. GFP-Ypt10p images were captured as a z-series. A deconvolved slice was then selected from the center of the cell and displayed. C) GFP-Ypt10p localization was determined in live cells serving as the sole copy in *ypt10ΔKAN/ypt10ΔKAN* diploid cells. Differential interference contrast (DIC) images were taken in one z plane. GFP-Ypt10p P_{YPT10} and FM4-64 fluorescence images were collected as a z-series. A deconvolved slice was then selected from the center of the cell from the TxRed and FITC channel from the same z plane and displayed.

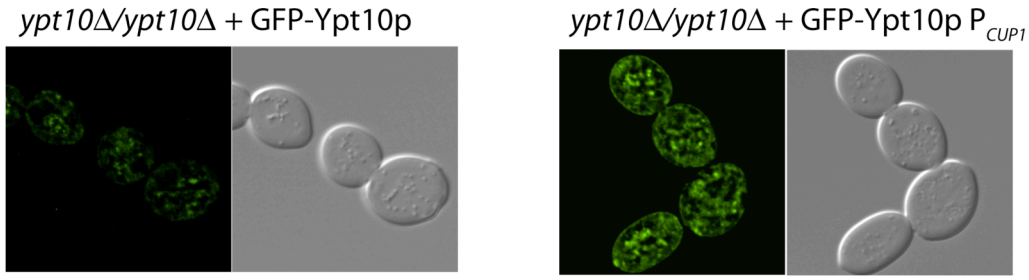
A)

GFP-Ypt10p functionality test



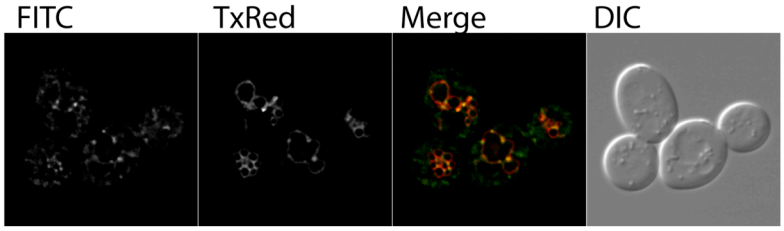
B)

GFP-Ypt10p localization



C)

GFP-Ypt10p, FM4-64 colocalization



membrane was observed and this was enhanced with the slight overexpresion when the GFP-tagged *YPT10* gene was driven from the P_{CUP1} promoter, which shows modest overexpression when grown in standard media without the exogenous addition of Cu^{++}.

Bioinformatic analysis did not reveal Ypt10p to be a member of a large subclass of Rab proteins, however, it is adjacent to the "Rab 20" group of endocytic Rab proteins [3, 15]. To examine the association of Ypt10p with organelles of the endocytic pathway, we performed colocalization analysis GFP-Ypt10p with vacuolar FM4-64. Double-labeled cells were visualized by fluorescence microscopy, with an optical slice taken from the cellular midsection to examine the coincidence of labels. FM4-64 stained vacuoles show good overlap with the GFP-Ypt10p-labeled puncta (Figure 3.5c) although the more peripheral, juxta-plasma membrane GFP-Ypt10p-labeled structures are not coincident with the vacuolar FM4-64. Overall, the localization of Ypt10p can be classified as peripheral endosomal/vacuolar.

Discussion

Localization analysis using GFP-tagged Rab GTPases identified the localization of the two uncharacterized Rab GTPases, Ypt10p and Ypt11p [3]. Ypt11p is found on ER membranes and functional analysis suggests a role in ER inheritance.

Ypt10p localizes to small puncta near the vacuole, and to a lesser extent, found regions near the plasma membrane. Overexpression of GFP-tagged *YPT10* is growth inhibitory, as is overexpression of *YPT10* alone suggesting GFP-Ypt10p functions similar to untagged Ypt10p [3, 17]. The localization suggests a role in the endocytic pathway; however, its impact on endocytic function remains unknown. Colocalization analysis with FM4-64 suggests the Ypt10p-positive puncta may localize to points on

the vacuole of fusing vacuoles. This localization may lead one to speculate on its function, perhaps regulating vacuolar fusion events between tethered vacuoles. An additional question to be answered in the future is how does overexpression of Ypt10p inhibit growth? This is a particularly interesting phenotype when compared to overexpression of the other Rab GTPases, as to my knowledge, overexpression of the other 10 Rab GTPases does not inhibit cell growth. Perhaps it simply creates a sink for Rab GDI and therefore inhibits recycling of the other Rab GTPases, or the growth inhibition is specifically related to Ypt10p function.

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Chapter 4. Elp1p, the yeast homolog of the FD disease syndrome protein, negatively regulates exocytosis independently of transcriptional elongation.[3]

Abstract

The activation of Rab GTPases is a critical focal point of membrane trafficking events in eukaryotic cells. However the cellular mechanisms that spatially and temporally regulate this process are poorly understood. Here we identify a null allele of *ELP1* as a suppressor of a mutant in a Rab guanine nucleotide exchange factor Sec2p. Elp1p was previously thought to be involved in transcription elongation as part of the Elongator complex. We show that *elp1Δ* suppression of $sec2^{ts}$ is not a result of reduced transcriptional elongation and that Elp1p physically associates with Sec2p. The Sec2p interaction domain of Elp1p is necessary for both Elp1p function and for the polarized localization of Sec2p. Mutations in human Elp1p (IKAP) are known to cause of Familial Dysautonomia (FD). Our results raise the possibility that regulation of polarized exocytosis is an evolutionarily conserved function of the entire Elongator complex and suggests that FD results from a dysregulation of neuronal exocytosis.

Introduction

Rab proteins are critical factors that regulate many different aspects of vesicular trafficking in eukaryotic cells [1-3]. As with other members of the Ras superfamily of GTPases, Rab proteins function as nucleotide-dependent molecular switches. The GDP-GTP switch is highly regulated. Guanine nucleotide exchange factors (GEFs) trigger GDP release and thus promote GDP-GTP exchange and activation. GTPase activating proteins (GAPs) stimulate GTP hydrolysis, returning the GTPase back to a GDP-bound inactive form.

[3] Portions of this work were published in Rahl, PB *et al.* Molecular Cell (2005) 17, *6*, pg. 841-853.

The yeast Rab protein Sec4p regulates the exocytosis of post-Golgi secretory vesicles [4]. Membrane traffic to the plasma membrane is a vectorial process coupled to cell-cycle events, thus Sec4p lies at a critical nexus of polarity establishment and maintenance events. Activated Sec4p binds the exocyst complex to target vesicle delivery to the bud tip. Sec4p activation is under the control of its GEF Sec2p [5], which itself is an effector for the Golgi Rab Ypt32p [6]. Sec2p is an essential protein that is recruited to sites of exocytosis [5], thereby targeting the Sec4p activation event and facilitating polarized exocytosis. Sec2p residues 450-541, distal to its GEF domain, are necessary for Sec2p localization. GEF localization and function in general appears to be a pivotal regulatory point for membrane trafficking events and the identification of proteins that influence these processes are of great interest in order to understand the signals that regulate membrane trafficking [6].

Elp1p is a member of a six-subunit complex (Elp1p, Elp2p, Elp3p, Elp4p, Elp5p, Elp6p) that has been identified in several independent studies. This complex is termed the Elongator as it was found to co-purify with the hyperphosphorylated form of RNA polymerase II [7, 8]. The mammalian homolog of Elp1p is the IKK-complex associated protein (IKAP), which is thought to function as a scaffold protein in the NFkB signal transduction pathway [9, 10]. Interest in the role of mammalian Elp1p IKAP has also been prompted by the discovery that mutations in IKAP are the cause of familial dysautonomia, a human autosomal recessive neurodegenerative disorder [11, 12].

The exact role of Elongator and the physiological relevance of its interaction with RNAPII are not well understood. Recent studies have shown that three subunits of the yeast Elongator complex, Elp1p, Elp2p and Elp3p, localize to the cytosol [13] and not to actively transcribing genes, leading to speculation as to additional Elongator functions [13, 14]. We report that Elp1p and Elongator negatively regulates polarized

secretion in budding yeast. Elp1p physically associates with, and is required for localization of the Sec4p GEF Sec2p. Association is dependent upon the localization domain of Sec2p and its dimerization but is independent of its exchange activity. We propose that Elp1p and the Elongator complex function in a cytosolic signal transduction pathway to regulate the localization of Sec2p and thereby the Rab activation event critical for polarized secretion.

Materials and methods

Yeast strains and Plasmid constructs – All yeast strains were manipulated using standard methods. *ELP1* was identified through a random insertion mutagenesis strategy [15] to identify genes that display synthetic interactions with *sec2-59*. The Elp1p truncation mutant ELP1*Δ*185 was created with the introduction of a stop codon after amino acid residue 1164. Elp1p-GFP-NLS was created by addition of the NH_2-terminal 33 residues of Cbp80p to the COOH-terminus of Elp1p-GFP. Elp1p-GFP-NLS^{mut} was created by mutation of the NLS amino acids K25A and R26A. Two-hybrid constructs used the vectors pAS2-1 and pACTII and were transformed into the Y190 strain.

Microscopy –Cells were examined with an Eclipse E600 (Nikon, Tokyo, Japan) equipped with a 60X objective and a 2.0X optovar. A Spot-RT monochrome charged-coupled device camera (Diagnostic Instruments, Stirling Heights, MI) with software version 3.5.4.1 was used for image capture. Anti-Act1p polyclonal antibody was used for F-actin immunofluorescence following the procedure described for anti-Tpm1p [16] and images processed with AutoDeblur software.

Co-purification and Western blotting – Bacterial lysates expressing either MBP-Sec2p or MBP were mixed with lysates from cells expressing His_6-Elp1p (residues 1165-1349), and incubated at 4˚C for 30 min in 50 mM Tris, 150 mM NaCl pH 8.0

with protease inhibitors. The mixtures were then purified on amylose resin columns (7.5 ml volume resin), washed with 10 column volumes of buffer and the MBP-fusion proteins eluted with 10 mM maltose. Fractions were analyzed by SDS-PAGE and Western blotting onto BioTrace 0.45µm PVDF membranes (Pall Corp.). Elp1p was detected with Chicken anti-Elp1p antibody, Sec2p with affinity-purified Rabbit anti-Sec2p, and MBP-fusion proteins with Rabbit anti-MBP antibody.

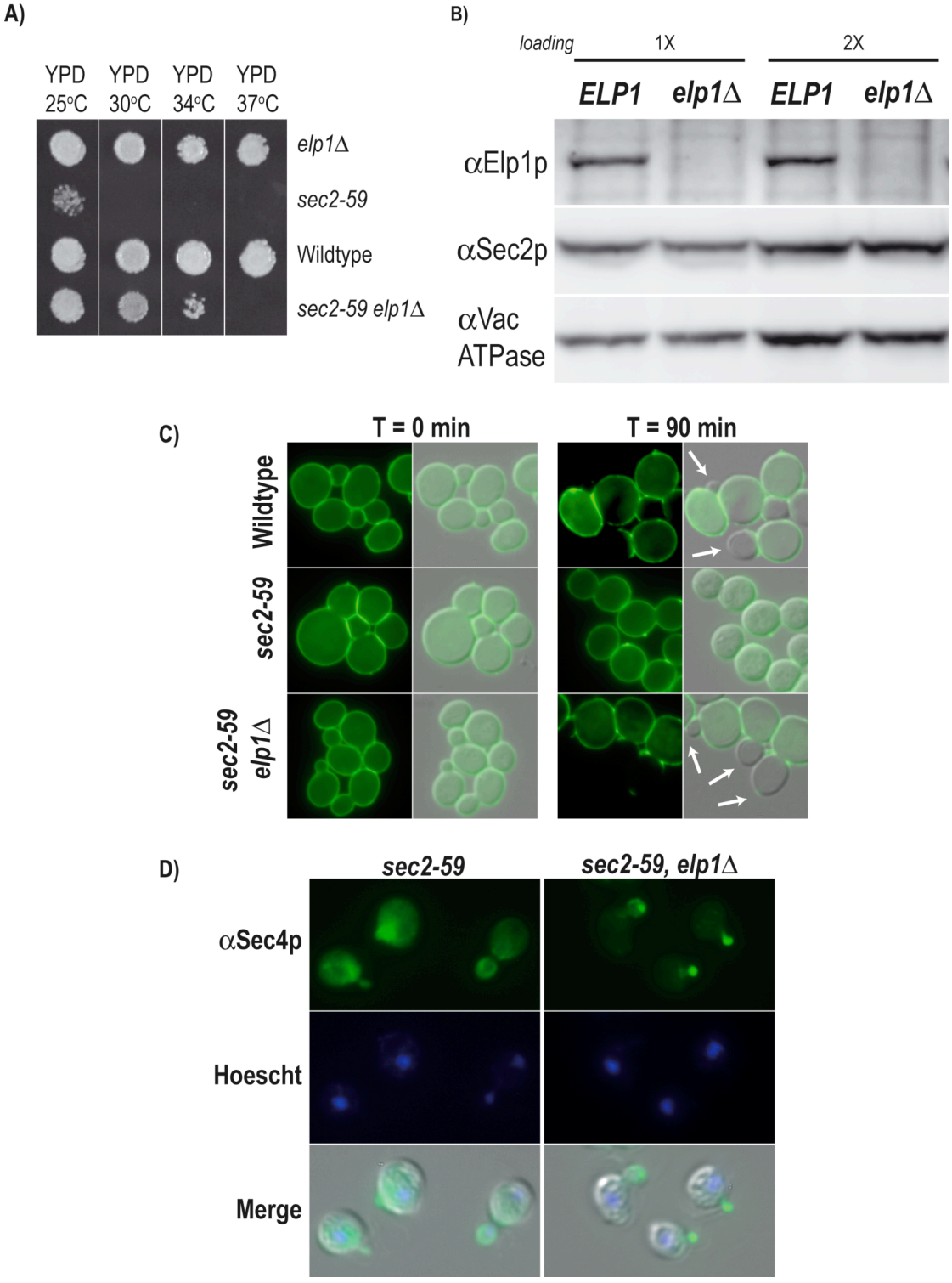
Results

Identification of a mutant that suppresses the *sec2-59* temperature sensitivity and secretion defect

sec2-59 is a temperature-sensitive allele of *SEC2* containing a stop codon after the first 374 residues [17]. The truncated protein encoded by *sec2-59* contains an intact GEF domain and has GEF activity equivalent to that of wild type Sec2p [18]. However, the Sec2p localization domain is missing from this mutant, and *sec2-59* cells are defective in secretion at restrictive temperature [5, 18]. To identify the factor or factors that could bypass the localization requirement and facilitate *sec2-59* function, we initiated a search for extragenic suppressors. Our search identified a single unlinked recessive suppressor. As can be seen in Figure 4.1a, *sec2-59* yeast grow at 25^{o}C but are dead at 30^{o}C. In contrast, isogenic *sec2-59 elp1*Δ cells grow at 34^{o}C although are still temperature-sensitive at 37^{o}C. The suppressing cells did not have altered Sec2p expression levels nor read-through of the stop codon (Figure 4.1b).

S.cerevisiae secrete in a polarized manner with new membrane inserted into the plasma membrane of the growing daughter cell. We used a FITC-ConA pulse

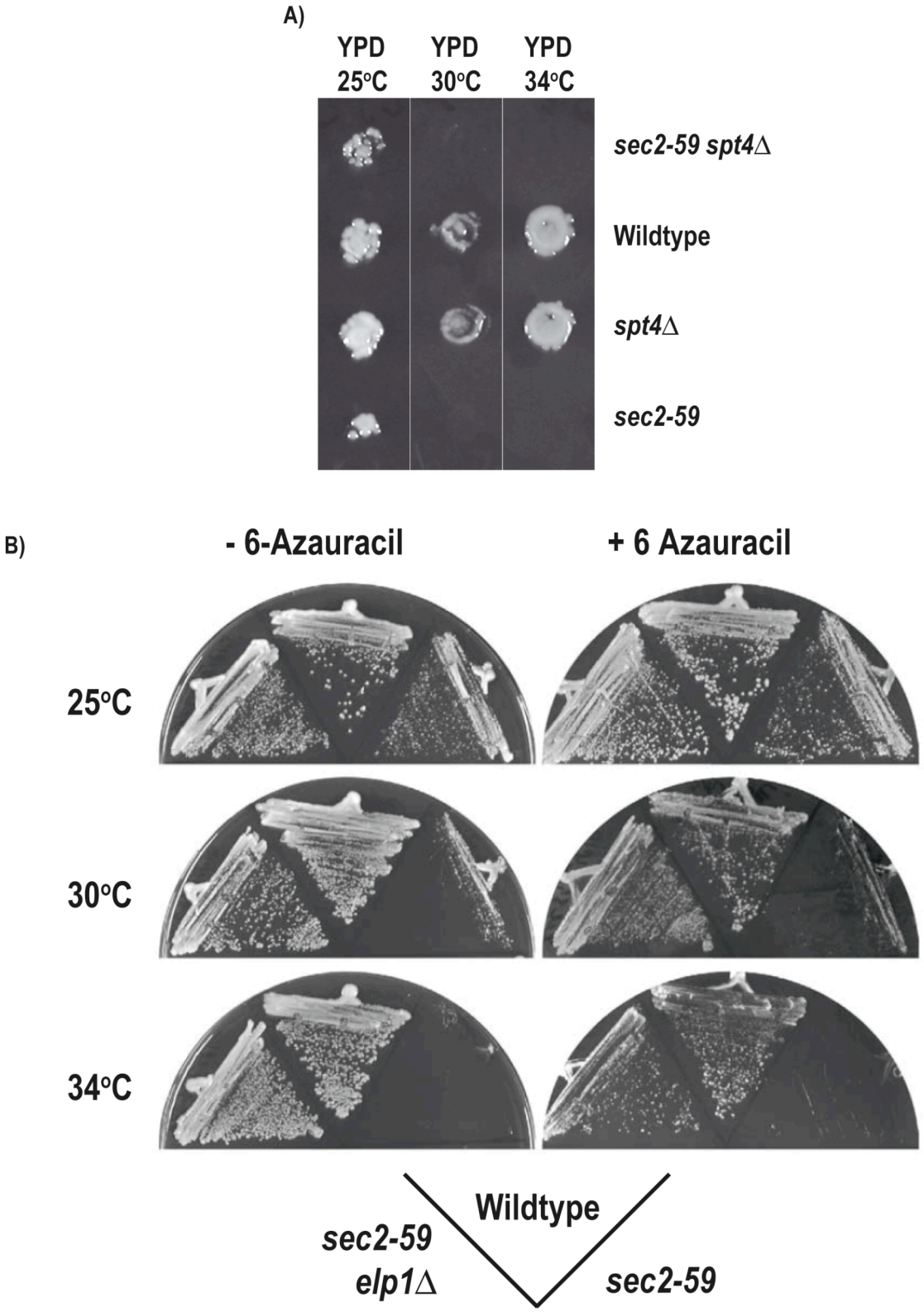
Figure 4.1. *elp1*Δ suppresses *sec2-59* temperature sensitivity and secretion defect A) Temperature sensitivity was assessed for isogenic *elp1*Δ*, sec2-59*, *sec2-59 elp1*Δ and wild type cells on YPD at 25°C, 30°C, 34°C and 37°C. B) Top panel: Lanes 1 and 2 show total lysates of wild type cells (representing ~0.1 and 0.2 OD_{600} cell units respectively) probed with Chicken antibodies against endogenous. Lanes 3 and 4 show equivalent amount of lysates prepared from *elp1Δ* cells. Bottom panel: Total lysates were prepared from wild type and *elp1Δ* cells and probed for Sec2p expression levels. Lanes 1 and 2 contain a loading of lysate representing ~0.1 OD_{600} cell units; Lanes 3 and 4 contain a loading of lysate representing ~0.15 OD_{600} cell units. The V-ATPase subunit detected by the 10D7 monoclonal antibody provides a loading control. C) Cells were monitored for polarized secretion with FITC-ConA labeling and pulse chase analysis. The plasma membrane was labeled with FITC-ConA at time zero followed by a 90 minute chase at restrictive conditions to allow for new membrane production. The plasma membrane of secretion-defective cells remains completely stained, while non-defective cells show the appearance of unmarked daughter cell membrane (white arrows). D) s*ec2-59* and *sec2-59 elp1*Δ cells were grown at room temperature to early log phase and temperature shifted to 30°C for one hour. Monoclonal anti-Sec4p immunofluorescence was used to determine localization of endogenous Sec4p. Hoechst 33258 was used to identify nuclei. Equivalent exposure length was used for all samples.


A)
YPD 25°C
YPD 30°C
YPD 34°C
YPD 37°C
elp1Δ
sec2-59
Wildtype
sec2-59 elp1Δ
B)
loading
1X
2X
ELP1
elp1Δ
ELP1
elp1Δ
αElp1p
αSec2p
αVac ATPase
C)
T = 0 min
T = 90 min
Wildtype
sec2-59
sec2-59 elp1Δ
D)
sec2-59
sec2-59, elp1Δ
αSec4p
Hoescht
Merge

chase assay to determine if suppression was due to a restoration of the *sec2-59* secretion defect. At the start of the experiment, the entire plasma membrane is labeled with FITC-ConA followed by a chase period under permissive or restrictive conditions. Polarized secretion can be monitored by the appearance of new unlabeled membrane in the daughter cell after a suitable chase period, as can be seen in the wild type cells in Figure 4.1c (white arrows). In the secretion defective (*sec2-59*) cells, exocytosis is blocked and the entire plasma membrane remains labeled. In the *sec2-59 elp1*Δ secretion mutant cells, new unlabeled membrane is deposited in the emerging daughter cell (white arrows), illustrating that the suppressor has restored the polarized secretion defect.

The substrate for Sec2p is the Rab GTPase Sec4p [5]. Sec4p normally localizes in a polarized manner to the site of secretion and can be observed in a distinctive pattern at the bud tip of emerging daughter cells. *sec2-59* cells are not impaired in GEF activity but are defective for proper localization of Sec2p and can no longer maintain a polarized distribution of Sec4p [5]. To investigate the mechanism of suppression, we asked if the suppressor could restore the polarized Sec4p localization that is defective in *sec2-59* cells. Figure 4.1d shows that Sec4p is mislocalized *sec2-59* cells, whereas in *sec2-59 elp1*Δ cells, Sec4p localizes normally and is concentrated at the sites of exocytosis, namely at the bud tip in small budded cells and at the neck region during cytokinesis. Thus, the suppressor acts on *sec2-59* through its ability to influence the polarity of the post-Golgi vesicle trafficking machinery.

Figure 4.2. *sec2-59* suppression is independent of transcription regulation
A) *spt4Δ*KAN^R cells were crossed with *sec2-59*. Temperature sensitivity was assessed for spores from a representative tetratype tetrad on YPD at 25^{o}C, 30^{o}C, 34^{o}C, and 37^{o}C. B) *sec2-59* and *elp1Δ sec2-59,* together with an isogenic wild type strain were transformed with pRS316 and tested on SC-U +/- 125 μg/ml 6-azauracil at 25^{o}C, 30^{o}C, and 34^{o}C.


A)
YPD 25°C
YPD 30°C
YPD 34°C
sec2-59 spt4Δ
Wildtype
spt4Δ
sec2-59
B)
- 6-Azauracil
+ 6 Azauracil
25°C
30°C
34°C
Wildtype
sec2-59 elp1Δ
sec2-59

elp1Δ suppression of *sec2-59* is not a result of reduced transcriptional elongation

Elp1p was originally identified as a component of the Elongator Complex that co-purifies with RNA polymerase II and functions to facilitate transcription elongation. We were surprised by our result identifying *elp1Δ* as a *sec2-59* suppressor as it is generally thought that the pathways controlling post-Golgi membrane trafficking and transcription elongation are unrelated. Although *sec* mutants are known to elicit transcriptional changes both through the arrest of secretion response (ASR) and the unfolded protein response (UPR), it is not clear how alterations in transcription could restore the detrimental impact of the *sec2-59* allele. To understand if the *elp1Δ* suppression of *sec2-59* is linked to changes in transcription, we asked if deletion of *SPT4,* another RNA PolII transcription elongation factor [19, 20], could similarly suppress *sec2-59* temperature sensitivity. Figure 4.2a illustrates that deletion of *SPT4* does not affect the temperature sensitivity of *sec2-59.*

The drug 6-azauracil (6-AU) has frequently been used as an indicator for involvement in transcriptional elongation. 6-AU causes a general reduction in the elongation phase of transcription and deletion or mutation of elongation factors in yeast are hypersensitive to 6-AU [21]. We used 6-AU to test if the defects in transcription elongation resulting from drug treatment, could cause suppression of *sec2-59*. Figure 4.2b shows that treatment with 125 μg/ml 6-AU did not alter the growth of *sec2-59* mutant cells, which remained temperature-sensitive, while the double mutant *elp1Δ sec2-59* grew at the restrictive temperature and was also unaffected by 6-AU treatment. These data show that *sec2-59* cannot be suppressed by reduced transcriptional elongation rates. Moreover, the lack of sensitivity of the double mutant *elp1Δ sec2-59* to 6-AU is further suggestive that PolII transcription is unaffected in this strain. We also tested to see if *sec2-59* could be suppressed by constitutive activation of the UPR or a general reduction in growth rates as a

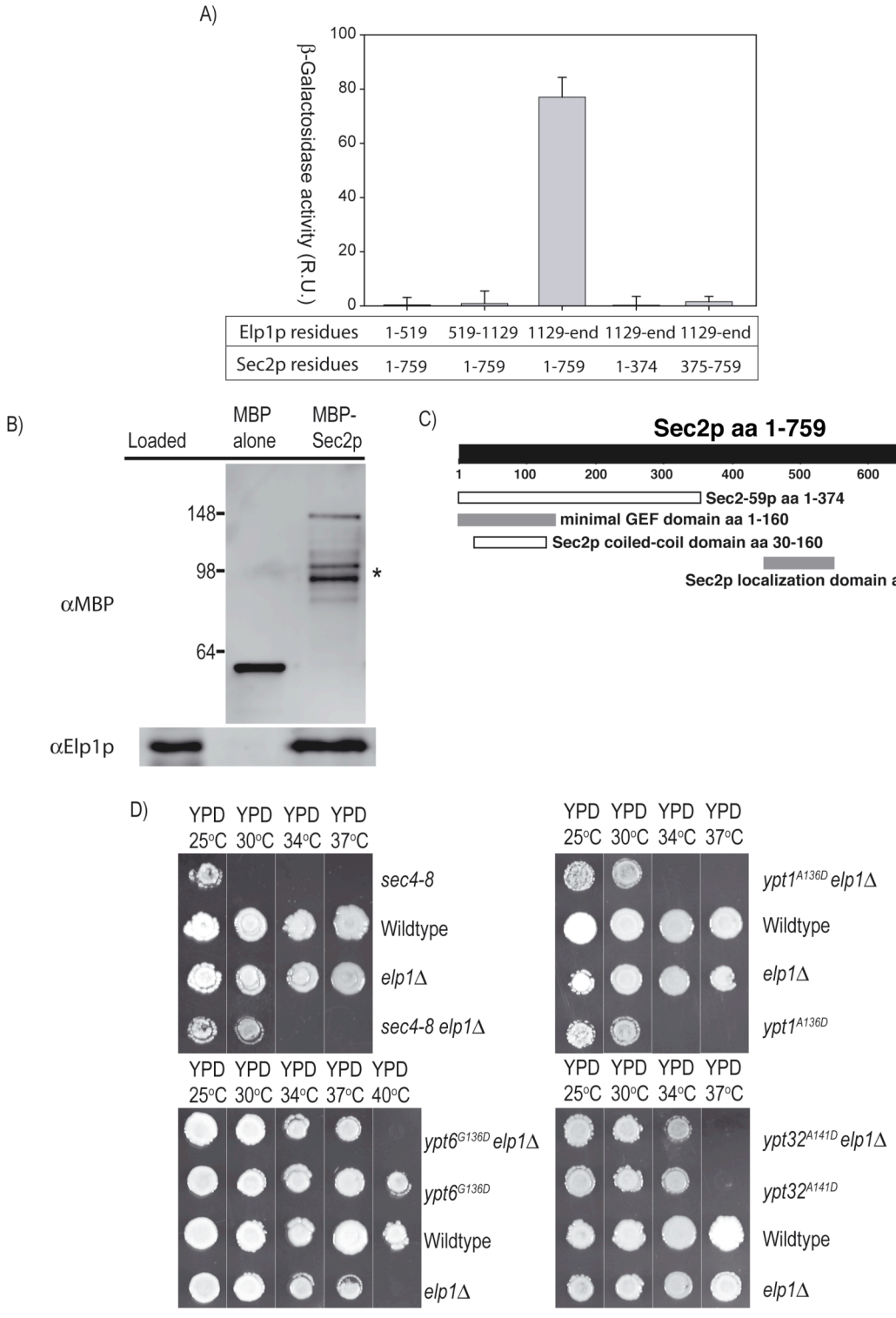
consequence of plating cells on different types of growth media, both with negative results (data not shown). Taken together, these data do not appear to link the role of Elp1p in *sec2-59* suppression to any decrease in transcription elongation rates or general growth defects, suggesting that the action of Elp1p is selective for Sec2p.

Elp1p physically associates with Sec2p

Because Elp1p appears to be acting in the same pathway as Sec2p, we sought to determine if a direct relationship exists between these two proteins. Initial screening using yeast two-hybrid analysis of Sec2p with Elp1p enabled us to identify a Sec2p interaction site at the COOH-terminus of Elp1p (Figure 4.3a). To determine if the association was direct or indirect, we expressed a recombinant Elp1p fragment containing the minimal Sec2p association domain (residues 1165-1349). Post-sonicate supernatants from Elp1p expressing bacteria were mixed with either Sec2p fused to MBP, or MBP alone. After incubation at 4˚C for 30 min, the lysates were passed over amylose resin, washed with 10 column volumes of buffer and the MBP proteins eluted by the inclusion of maltose in the buffer. Comparison of the eluates (Figure 4.3b) from MBP-Sec2p and MBP alone expressing cells demonstrates that Elp1$^{1165\text{-}1349}$p co-purifies with Sec2p indicating a direct association of these two proteins. The localization-defective *sec2-59* allele that is suppressed by Elp1p comprises the NH_2-terminal 374 residues of Sec2p. Sec2-59p lacks the localization domain but contains the GEF domain together with a coiled-coil region and a binding site for GTP-bound Ypt32p (Figure 4.3c). Elp1p did not interact with the residues 1-374 or 375-end of Sec2p indicating that it requires features in both the NH_2- and COOH-terminal domains of Sec2p for interaction.

Sec2p is an effector for the Golgi Rab Ypt32p [6] in addition to regulating activation of the post-Golgi Rab Sec4p. If Elp1p is acting in a pathway that regulates

Figure 4.3. Elp1p physically associates with Sec2p.
A) Y2H analysis shows Elp1p COOH-terminus and Sec2p interaction. Pairs of constructs as indicated were co-expressed in the reporter strain Y190 and b-galactosidase activity (arbitrary units) measured. B) A recombinant COOH-terminal fragment of Elp1p co-purifies with MBP-Sec2p. Lysates from bacteria expressing His_6-Elp1p (residues 1165-1349) were mixed with either MBP or MBP-Sec2p (full length) as described in the text. The mixtures were then purified on amylose resin and the associated MBP-fusion proteins eluted with 10 mM maltose. Fractions were analyzed by Western blot for the presence of Elp1p and MBP (after 100-fold dilution). Lane 1 represents 0.1% of the total post-sonicate supernatant expressing His_6-Elp1p. Lanes 2 and 3 show the elution fractions from either MBP alone (Lane2) or MBP-Sec2p (Lane3). Asterisk indicates degradation product from MBP-Sec2p. C) Linear depiction of the primary amino acid sequence of Sec2p (759 residues total) outlines the minimal GEF domain (residues 1-160), the coiled-coil region (residues 30-160), the localization domain (residues 450-541) and the protein encoded by the localization-defective temperature sensitive allele *sec2-59* (residues 1-379). The binding site for GTP-bound Ypt32p is contained within Sec2-59p, residues 1-379. D) *elp1Δ* cells were crossed with $sec4^{G147D}$, $ypt6^{G139D}$, $ypt1^{A136D}$, or $ypt32^{A141D}$ *ypt31D* (note that the $ypt32^{A141D}$ mutation is only apparent in the background where the functionally redundant *ypt31* is deleted). Temperature sensitivity was assessed for spores from a representative tetratype tetrad on YPD at 25°C, 30°C, 34°C, 37°C and 40˚C.

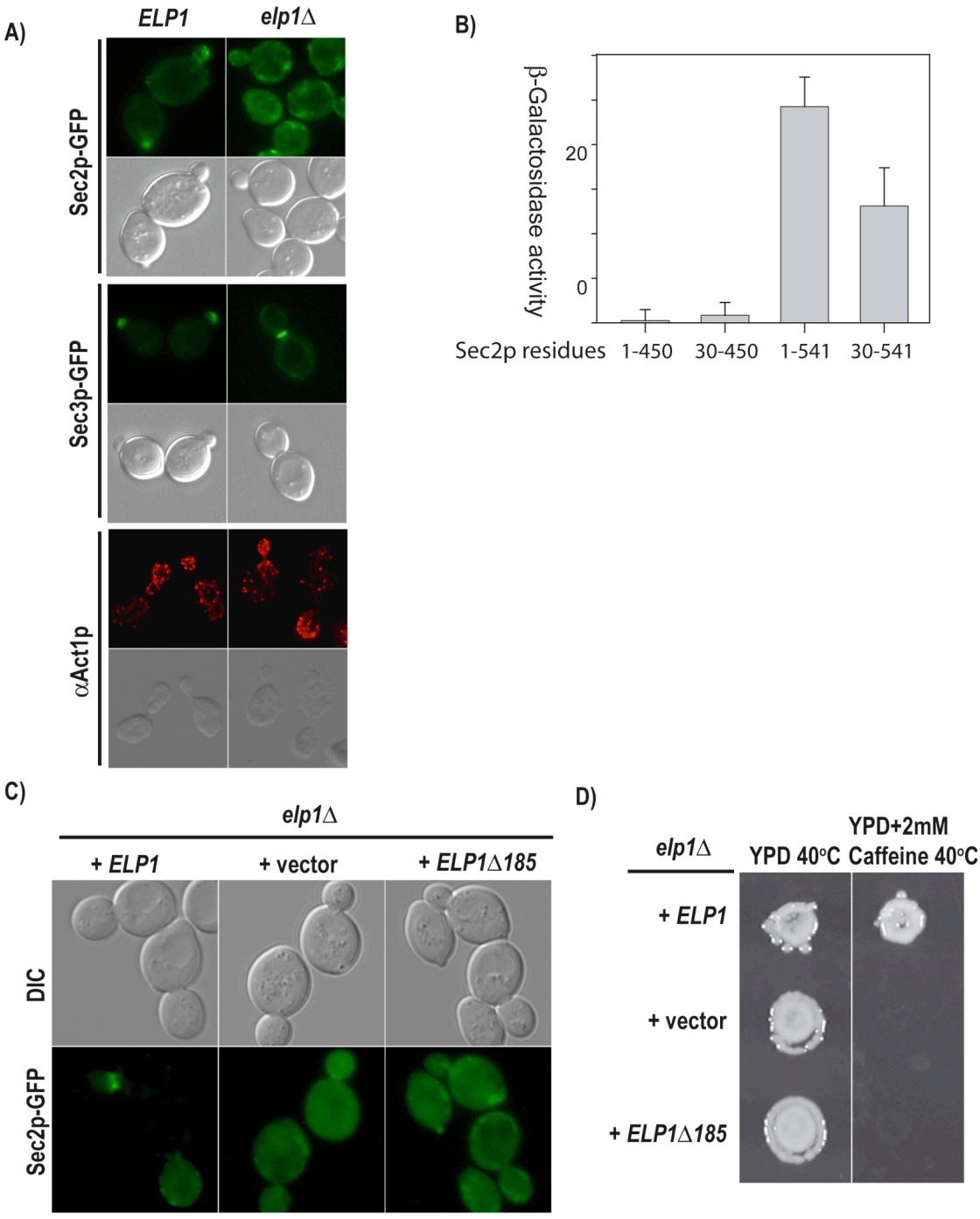

A)
β-Galactosidase activity (R.U.)
100
80
60
40
20
0
Elp1p residues 1-519 519-1129 1129-end 1129-end 1129-end
Sec2p residues 1-759 1-759 1-759 1-374 375-759
B)
Loaded
MBP alone
MBP-Sec2p
148
98
64
*
αMBP
αElp1p
C)
Sec2p aa 1-759
1 100 200 300 400 500 600 700
Sec2-59p aa 1-374
minimal GEF domain aa 1-160
Sec2p coiled-coil domain aa 30-160
Sec2p localization domain aa 450-541
D)
YPD 25°C YPD 30°C YPD 34°C YPD 37°C
sec4-8
Wildtype
elp1Δ
sec4-8 elp1Δ
YPD 25°C YPD 30°C YPD 34°C YPD 37°C
ypt1A136D elp1Δ
Wildtype
elp1Δ
ypt1A136D
YPD 25°C YPD 30°C YPD 34°C YPD 37°C YPD 40°C
ypt6G136D elp1Δ
ypt6G136D
Wildtype
elp1Δ
YPD 25°C YPD 30°C YPD 34°C YPD 37°C
ypt32A141D elp1Δ
ypt32A141D
Wildtype
elp1Δ

Ypt32p function, we reasoned that the deletion of *ELP1* should suppress *ypt32* mutants in an analogous fashion to its suppression of *sec2-59* mutants. Conversely, if Elp1p regulates the downstream events of Sec2p function the deletion of *ELP1* should also suppress *sec4* mutants. To distinguish these possibilities, we made use of a series of conditional mutants in exocytic Rab proteins that contain identical point mutations in a small, uncharged residue (alanine or glycine) on the surface of helix 5. For all the exocytic Rab proteins Sec4p, Ypt1p, Ypt32p and Ypt6p, this single mutation has been demonstrated to confer a tight and rapid block of Rab function at the restrictive temperature [4, 22-24]. *elp1D* in combination with any Rab mutant in this series has no effect except with *sec4-8* (*sec4*^{G147D}), where a distinct suppression could be observed (Figure 4.3d). These data suggest that Elp1p acts via Sec2p in a pathway that is downstream of Golgi Ypt32p function and results in increased Sec4p Rab GTPase activity.

Elp1p is required for polarized localization of Sec2p

Our genetic data suggested that the removal of Elp1p results in an increase in the Rab GTPase Sec4p activity. Because Elp1p can physically interact with the Rab exchange factor Sec2p, we wished to understand how its loss could impact Sec4p functionality. The loss of polarity of the vesicular trafficking machinery in *sec2-59* cells is thought to result from loss of normal Sec2p localization because the mutant *sec2-59* allele lacks a localization domain. We therefore examined any possible involvement of Elp1p in localization of wild type Sec2p. Wild type Sec2p, although a cytosolic protein, is normally concentrated in a distinctive polarized manner to the sites of exocytosis [18]. Fluorescence microscopy (Figure 4.4a) revealed that Elp1p is required for this polarized localization, because deletion of *ELP1* causes Sec2p-GFP to be dispersed throughout the cell. This effect is specific to Sec2p because deletion of

Figure 4.4. *ELP1* is required for localization of Sec2p.
A) GFP-Sec2p and Sec3p-GFP were transformed into wild type and *elp1Δ* cells. Transformants were grown to log phase at room temperature and then shifted to 37°C for 1h prior to live cell microscopy. Bottom panel: Cells grown as for top panel, processed for actin (αAct1p) immunofluorescence. B) Y2H analysis of Elp1p association with Sec2p constructs lacking the 30 residues and the NH_2-terminus and localization domain. C) GFP-Sec2p was transformed into *elp1Δ* cells containing vector only, ELP1*Δ*185, or *ELP1* wildtype plasmids. Transformants were grown to log phase at room temperature and shifted to 37°C for 1h prior to live cell microscopy. D) *elp1Δ* cells were transformed with vector only, *ELP1*, or the truncated ELP1*Δ*185 construct and tested on YPD and YPD+2mM caffeine at 40°C. Wild type *ELP1* and empty vector served as positive and negative controls respectively.


A)
ELP1
elp1Δ
Sec2p-GFP
Sec3p-GFP
αAct1p
B)
β-Galactosidase activity
20
0
Sec2p residues 1-450 30-450 1-541 30-541
C)
elp1Δ
+ ELP1
+ vector
+ ELP1Δ185
DIC
Sec2p-GFP
D)
elp1Δ
YPD 40°C
YPD+2mM Caffeine 40°C
+ ELP1
+ vector
+ ELP1Δ185

ELP1 does not effect the localization of Sec3p, a polarity landmark and a subunit of the exocyst complex [25, 26], suggesting that the polarity machinery upstream of Sec2p remains unaffected. A polarized actin cytoskeleton is also required for spatial targeting of exocytosis and disruptions of the actin cytoskeleton asymmetry result in Sec2p mislocalization [5]. We therefore examined the possibility that Elp1p affected Sec2p localization indirectly via effects on actin organization. The ultrastructure of the actin cytoskeleton of wild type and *elp1Δ* cells was examined by anti-Act1p immunofluorescence microscopy. The resulting images (Figure 4.4a), show no observable differences in organization of the actin cytoskeleton. These data indicate that Elp1p influences Sec2p localization in a manner independent of actin organization.

If Elp1p acts to correctly localize Sec2p *in vivo*, we expected that it would be dependent on the COOH-terminus of Sec2p that contains the localization domain (residues 450-541) [18], see Figure 4.3b. We accordingly made constructs comprising Sec2p residues 1-450 and 1-541. Figure 4.4b shows that Elp1$^{1164\text{-}1349}$p interacts with Sec2p residues 1-541, which is the minimal length of Sec2p that retains correct localization, but not with Sec2p residues 1-450 which does not correctly localize *in vivo*. Thus Elp1p is required for Sec2p localization *in vivo* and physically associates with Sec2p in a manner dependent upon the previously identified localization domain of Sec2p. The localization domain of Sec2p is necessary but not sufficient for Elp1p interaction as a construct containing the COOH-terminus of Sec2p, the portion of Sec2p missing in the *sec2-59* allele, does not show Elp1p interactions (Fig. 4.3a). Two additional features have been defined in the NH_2-terminus of Sec2p; the GEF domain, (residues 1-160), and a coiled-coil region that mediates dimerization [17], (residues 30-160), see Fig. 3B. To determine the contribution of each of these features to Elp1p interaction, we removed 30 residues from the NH_2-terminus in order to disrupt the

GEF domain without affecting the integrity of the coiled-coil domain. The resultant Sec2p protein is capable of self-self interaction (data not shown), however it no longer has exchange activity towards Sec4p (data not shown). A Sec2p construct of residues 30-541 still showed interactions with Elp1p indicating that an active exchange domain of Sec2p is not required for Elp1p association (data not shown). These data demonstrate that the physical association between Elp1p and Sec2p requires the Sec2p localization domain and an intact coiled-coil motif. Taken together, our data suggests that Elp1p serves to impact Sec2p activity indirectly via spatial regulation of the exchange factor.

The Sec2p-interaction domain of Elp1p is necessary for Elp1p function and localization of Sec2p

As our data show that the COOH-terminal 185 residues of Elp1p interact with Sec2p and that interaction requires the localization domain of Sec2p, we next created a truncated form of Elp1p, ELP1*Δ*185, lacking the Sec2p-interaction domain in order to determine if such a mutant could rescue the mislocalization of Sec2p observed in *elp1Δ* cells. Figure 4.4c illustrates that the truncated version of Elp1p, lacking the Sec2p-interaction domain, cannot restore wild type Sec2p localization compared to full length Elp1p. Thus our data reveal that the COOH-terminal 185 residues of Elp1p are both necessary for Sec2p interaction, and required for the correct localization of Sec2p *in vivo*.

Elp1p is a large protein of 1349 amino acids of which the minimal Sec2p interaction domain comprises the COOH-terminal 185 residues. In order to understand the contribution of the Sec2p interaction domain to overall Elp1p function, we asked if the ELP1*Δ*185 construct was able to provide *ELP1* function and complement *elp1Δ*. Elp1p function was assessed by the ability of the plasmid to complement an *elp1Δ*

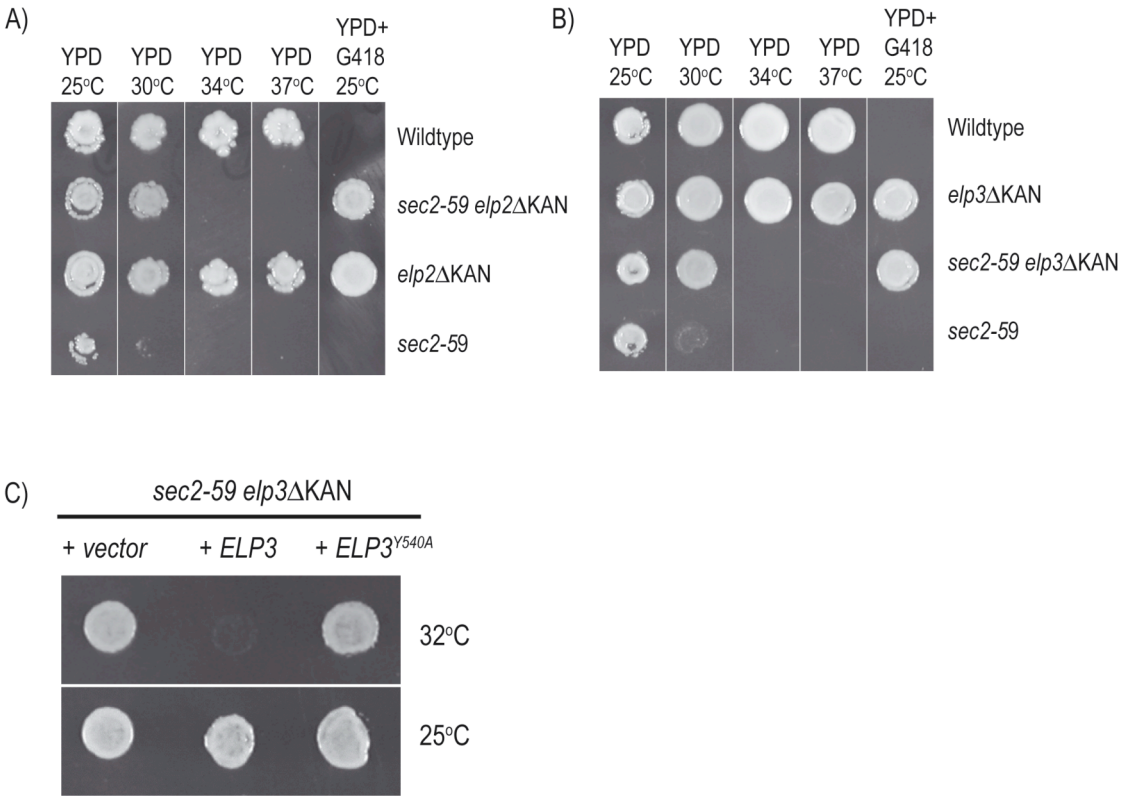


Figure 4.5. Genetic interactions between *sec2* and other elongator complex members. A) Deletion of the *ELP2* and *ELP3* genes results in a suppression of *sec2-59.* A representative tetratype tetrad is shown. G418-containing media indicates possession of the respective *elp* gene knockout. B) Acetyltransferase activity of Elp3p is required for suppression of *sec2-59.* The double mutant *elp3D* sec2-59 was transformed with plasmids as indicated and plated on selective media at various temperatures.

strain that is temperature-sensitive on caffeine-containing media. Figure 4.4d shows that the ELP1*Δ*185 construct in *elp1Δ* cells is unable to provide growth at 40°C on YPD+2mM caffeine, by comparison with an identical construct of full length Elp1p that is fully functional. These data demonstrate that the portion of Elp1p that interacts with Sec2p is not dispensable for Elp1p functionality, suggesting that Sec2p interaction and localization represent the primary role of Elp1p.

Genetic interactions between *sec2*^{ts} and other Elongator Complex members

Our genetic, biochemical and cell biological data indicate a role for Elp1p in polarized exocytosis. In order to understand if this is a unique role for Elp1p in addition to other possible functions as part of the Elongator Complex, we examined interactions between *sec2*-59 and genes encoding other Elongator complex subunits. We focused on Elp2p and Elp3p as these proteins exist as part of a more stable core sub-complex with Elp1p [27]. Figure 4.5a shows the growth of colonies from spores of a representative tetratype tetrad from each of these crosses. Either *elp2Δ* or *elp3Δ* can suppress temperature sensitivity of *sec2-59,* although the level of suppression is not as pronounced as with *elp1Δ.* These genetic data implicate other members of the Elongator Complex in polarized exocytosis and suggest that Elp1p does not act alone in its impact on polarized secretion but probably functions in the context of its association with other members of the Elongator complex.

The Elp3p subunit of Elongator has a demonstrated biochemical activity, that of an acetyltransferase [28]. Because a deletion of *ELP3* can restore the growth of *sec2-59* cells at restrictive temperatures, we reasoned that if the acetyltransferase activity of Elp3p is required for the *sec2*^{ts} suppression phenotype, a mutation which abolishes this activity would result in the same phenotype as a null allele. For this experiment, we made use of a single point mutation in a region conserved among acetyltransferases that has been demonstrated to abolish the acetyltransferase activity of Elp3p [29]. *elp3Δ sec2-59* cells were transformed with plasmids encoding either *ELP3*, *elp3*^{Y540A} or vector only controls. Transformants were plated onto selective media, to ensure retention of the plasmid, and at different temperatures to determine the ability of the plasmids to maintain or abolish the suppression of *sec2-59*. These data are shown in Figure 4.5b. *elp3Δ sec2-59* cells transformed with vector control still showed growth at restrictive temperature, which was eliminated in the presence of

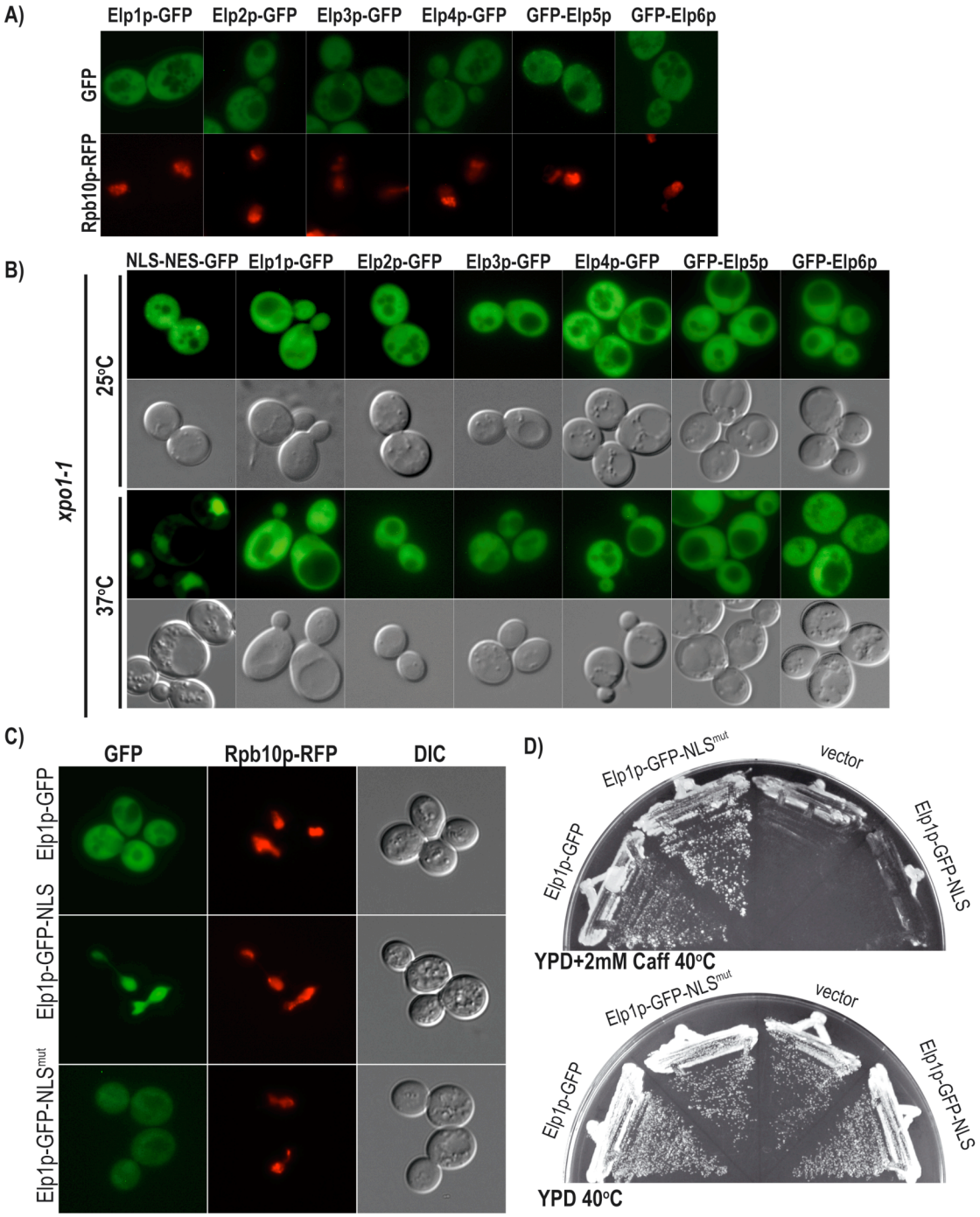
the wild type *ELP3* plasmid. Cells transformed with the *elp3*^{Y540A} containing plasmid gave a phenotype resembling the vector control indicating that the ability of the wild type *ELP3* gene to regulate Sec2p function requires its acetyltransferase activity.

Elongator subunits localize to the cytosol at steady-state and do not shuttle between the cytosol and nucleus

The role of the Elongator complex in facilitating transcriptional elongation is presumably a nuclear function [7]. The genetic and biochemical data that indicated a role for Elongator in membrane trafficking led us to explore the localization of each subunit. Although Elongator can physically associate with RNA PolII, a nuclear resident protein, it was previously shown that myc-tagged Elp1p, Elp2p and Elp3p are located in the cytosol [13]. We also examined the localization of the three remaining Elongator subunits, Elp4p, Elp5p and Elp6p. Functional copies of a GFP-tagged Elongator subunit can rescue a deletion of the respective *ELP* gene from a lethal phenotype at 40^{o}C on YPD+2mM caffeine. This test was used to identify functional GFP-tagged copies of each Elongator subunit (data not shown). We examined the steady state distribution of Elp1p-GFP, Elp2p-GFP, Elp3p-GFP, Elp4p-GFP, GFP-Elp5p, and GFP-Elp6p by fluorescence microscopy where each construct represented the only copy of the respective gene. A fusion of the RNA PolII subunit Rpb10p, to RFP was used as a marker for the nucleus in these experiments. All six Elongator subunits showed a steady-state cytosolic localization (Figure 4.6a).

A protein that is resident in the cytosol under steady state conditions may still have an active role in the nucleus if it transits between these two compartments and is exported from the nucleus at a higher rate than its import. The nuclear localization of such a shuttling protein becomes readily apparent under situations where nuclear export is blocked and the protein accumulates in the nucleus. We used mutant nuclear

Figure 4.6. Elongator Complex subunits localize to the cytosol at steady-state and do not shuttle between the nucleus and cytosol. A) Functional copies of GFP-tagged Elongator subunits were co-transformed into their respective diploid knockout strain together with Rpb10p-RFP as a nuclear marker. Co-transformants were grown at room temperature to log phase for live cell microscopy. B) Functional copies of GFP-tagged Elongaator subunits and NLS-NES-GFP_2 were transformed into the *xpo1-1* strain containing a temperature sensitive allele of the CRM1 nuclear export receptor. Transformants were selected and grown to log phase at permissive temperature. Cells were either maintained at permissive temperature or shifted to restrictive temperature (37^oC) for 1h and GFP localization was assessed to determine the nuclear-cytoplasmic shuttling properties of the tagged protein. C) Plasmids encoding GFP-tagged Elp1p alone, and with the addition of either a nuclear localization signal or a mutant version of the NLS, were introduced into *elp1Δ* cells. Constructs were assessed for localization and the ability of the plasmid to complement the caffeine and temperature sensitive phenotype of *elp1Δ* cells.


A)
Elp1p-GFP
Elp2p-GFP
Elp3p-GFP
Elp4p-GFP
GFP-Elp5p
GFP-Elp6p
GFP
Rpb10p-RFP
B)
NLS-NES-GFP
Elp1p-GFP
Elp2p-GFP
Elp3p-GFP
Elp4p-GFP
GFP-Elp5p
GFP-Elp6p
xpo1-1
25°C
37°C
C)
GFP
Rpb10p-RFP
DIC
Elp1p-GFP
Elp1p-GFP-NLS
Elp1p-GFP-NLS^mut
D)
Elp1p-GFP-NLS^mut
vector
Elp1p-GFP
Elp1p-GFP-NLS
YPD+2mM Caff 40°C
YPD 40°C

exportin strains, *xpo1-1, cse1-2,* and *msn5Δ,* to determine if the Elongator complex shuttles between the cytosol and the nucleus [30-33]. *XPO1* is the major nuclear exportin in yeast and is a member of the CRM1 karyoferin family [30]. As can be seen in Figure 4.6b, at permissive temperature in *xpo1-1* strains, a NLS-NES-GFP control construct and the six Elongator subunits have a diffuse localization throughout the entire cell. Upon shifting to restrictive temperature for one hour, the NLS-NES-GFP construct now accumulates in the nucleus while the Elongator subunits remain cytosolic. Thus the Elongator subunits do not appear to be actively shuttling between the nucleus and cytosol using the *CRM1* pathway, the primary nuclear export pathway for proteins. Similar analyses using *cse1-2* and *msn5Δ* strains, nuclear exportins with a more limited substrate range, revealed that Elongator does not shuttle between the cytosol and nucleus using these alternate pathways (data not shown). These data indicate that Elongator does not constitutively shuttle between the nucleus and cytosol using the nuclear export mediators tested.

A protein that does not constitutively shuttle between the nucleus and cytosol may still translocate to and function in the nucleus in response to a specific stimulus. We tested several conditions to determine if we could identify a situation that would change the steady state distribution of Elongator complex subunits. However, Elongator subunits remained cytosolic under all conditions tested, and even conditions where Elongator proteins are known to be essential (2mM caffeine at 40˚C), failed to elicit a nuclear localization response from any Elongator complex subunit (data not shown).

Nuclear compartment restricted Elp1p cannot complement *elp1Δ*

In order to determine if the cytoplasmic localization of Elp1p is critical for function, we asked if we could confine Elp1p to the nuclear compartment and still

retain function. To restrict Elp1p localization, we added a nuclear localization sequence to the COOH-terminus together with GFP to monitor *in vivo* localization. Figure 4.6c demonstrates that Elp1p-GFP-NLS resides in the nucleus under steady state conditions. As controls, we used an Elp1p construct tagged with GFP alone, and an Elp1p-GFP-NLS^{mut} construct that contains a double point mutation (2/33 amino acids) in the NLS that abolishes its ability to act as a nuclear localization signal [34]. Neither control construct localizes to the nucleus. We then asked if the nuclear-resident Elp1p could provide function. Elp1p function is essential for viability at 40˚C on rich media containing 2 mM caffeine. Figure 4.6d shows that Elp1p-GFP-NLS cannot provide Elp1p function, in contrast to the cytoplasmically localized control constructs Elp1-GFP and Elp1p-GFP-NLS^{mut}. The inability of Elp1p-GFP-NLS to provide Elp1p function is unlikely to be related to any blockade of the COOH-terminus of Elp1p, as Elp1p can be successfully tagged at the COOH-terminus of the protein (although not its NH_2-terminus, data not shown), and the Elp1p-GFP-NLS^{mut}, which does complement *elp1Δ,* provides a COOH-terminal tag of the same size. These data suggest that the reason Elp1p-GFP-NLS is unable to provide function is due to its nuclear localization, further supporting the argument that there is a functional requirement for Elp1p in the cytosol and not in the nucleus.

Discussion

Elp1p regulates localization of the GEF Sec2p

We initially identified *ELP1* as a gene whose deletion suppresses a localization-defective temperature-sensitive allele of the Rab GEF *SEC2*. *elp1Δ* is not a bypass suppressor as its deletion does not eliminate the requirement for *SEC2*, rather, its genetic properties suggest that Elp1p is negatively regulating Sec2p-

dependent polarized secretion. The *sec2-59* mutant is epistatic to the *elp1* deletion allele because the *sec* temperature sensitivity phenotypes are observed in double mutants. Suppression by epistasis occurs when two mutations cause opposite phenotypes and one mutation suppresses the other, indicating two proteins functioning in the same pathway. The ability of *elp1Δ* to suppress *sec2-59* led us to explore a physical association between the two proteins, which revealed that the COOH-terminus of Elp1p contains a Sec2p interaction site. Supporting the genetic and biochemical data suggesting a biologically relevant interaction between these two proteins, we demonstrate *in vivo* that Elp1p is required for proper localization of Sec2p, that localization requires the Sec2p-interaction domain of Elp1p and also that the COOH-terminal Sec2p-interaction domain is necessary for Elp1p to complement *elp1Δ*. Removal of the COOH-terminal Sec2p interaction domain from Elp1p cannot rescue the caffeine sensitivity phenotype of *elp1Δ*. Deletion of the COOH-terminus of Elp1p does not affect its ability to associate with other subunits of the Elongator complex [35], suggesting that the ELP1*Δ*185 construct is not functional because it fails to interact with Sec2p and that this interaction represents the critical function of Elp1p. The Elongator complex comprises two sub-complexes consisting of Elp1/2/3p and Elp4/5/6p respectively [27, 36]. All members of the elongator complex are thought to work together in vivo as they share similar phenotypic profiles [36, 37]. Deletion of either *ELP2* or *ELP3* can also suppress *sec2-59* suggesting that regulation of Sec2p localization is not an independent function of Elp1p, but rather that Elp1p is acting in the context of the Elongator complex.

An additional or alternative role for Elp1p and Elongator?

The genetic, biochemical and cell biological data we report in this study, strongly suggest a role for Elp1p and the Elongator complex in late stages of vesicular

trafficking. *ELP1* was originally identified as a gene whose deletion confers resistance to the *K. lactis* killer toxin [38]. Genes encoding all six Elongator subunits share this phenotype, but because the toxin blocks cell cycle progression in a poorly understood manner, it is not helpful for ascribing protein function. Subsequent studies identified Elp1p as a component of the transcription initiation and elongation apparatus [7]. Elp1p co-purifies with the elongating form of RNAPII, however *elp1Δ* strains show no sensitivity to 6-AU, in contrast to other genes involved in transcriptional elongation.

Our results suggest that the *elp1Δ* suppression of *sec2-59* cannot be achieved through a general reduction in transcription. Is Elp1p "moonlighting" in two different roles? A transcriptional role for Elongator has also been questioned by several previously published reports. Efforts by Krogan *et al.* failed to identify an interaction between the phosphorylated form of RNA PolII and Elongator, as Elp1p, Elp2p, Elp3p and Elp5p were unable to pull down RNA PolII [14]. A systematic, *in vivo* exploration of the recruitment of elongation factors to promoters and open reading frames of activated genes did not find Elongator in association with actively transcribing genes [13]. Our data also argues against a nuclear role for Elp1p, as Elp1p does not enter the nucleus, and its phenotype can be accounted for by its Sec2p interaction domain. Furthermore, we were unable to trap any Elongator subunits in the nucleus under conditions that cause the accumulation of bona fide nucleo-cytoplasmic shuttling proteins. Addition of a NLS to Elp1p caused the protein to become nuclear resident and rendered it unable to complement *elp1Δ*, in contrast to an equivalent construct with a mutation in the NLS that remained cytoplasmic. Although we cannot completely exclude the possibility that a tiny fraction of Elp1p does enter the nucleus to stimulate transcription under circumstances yet to be determined, these data strongly suggest that the essential role of Elp1p is cytoplasmic and not nuclear. Furthermore, a number

of high-throughput screens have identified synthetic lethal relationships between *ELP* genes and genes involved in cell polarity determination and/or maintenance, supporting a role for Elongator in polarized exocytosis [39, 40]. These data collectively support an active requirement for the Elongator complex in the establishment or maintenance of cell polarity.

Mechanism of Elongator action

Mammalian homologs of Elp1p and Elp2p have been identified as participants of the NFκB and STAT (signal transducer and activator transcription factor) kinase signal transducing networks. The mammalian homolog of Elp1p, IKAP, was identified as a protein binding to IκK, the IκB kinase [9, 41, 42] and also as a JNK associated protein [43]. The mammalian homolog of Elp2p is StIP1, a protein that binds to several STATs and also Janus kinases [44]. StIP1 contains WD-40 motifs and has been proposed to act as a scaffold for the assembly of a signaling complex. Although the primary sequence homology between these proteins and Elp1/2p is significant enough for them to be considered as genuine orthologs, signaling pathways such as the NF-κB and the Janus kinase/STAT pathway are not conserved in *S. cerevisiae*. Thus the Elongator complex may represent a signaling scaffold that is conserved between evolutionarily divergent cells that can adapt to different regulatory inputs and outputs. One possibility raised by our data is that regulation of polarized exocytosis is an evolutionarily conserved function of the Elongator complex and it is the failure to spatially regulate exocytosis in neurons that underlies the defect of Familial Dysautonomia.

Currently Elp3p is the only subunit with a recognized biochemical activity, protein acetylation. Elp3p is capable of acetylating all four histones in vitro [28], although histones may not be the physiologically relevant substrates. Reversible

acetylation regulates important biological processes in addition to histone metabolism and may play a much broader function in cell signaling than previously appreciated [45]. Clearly it will be important to establish the physiological *in vivo* substrates of Elp3p and identify the relevant deacetylase entity(ies).

In conclusion, the balance of evidence we present argues that the yeast Elongator complex acts through a cytoplasmic signal transduction pathway to regulate polarized exocytosis through localization of the GEF Sec2p. The role of Elp1p and Elongator in exocytosis is functionally distinct from any involvement in transcriptional elongation, and most likely represents the bona fide function of Elongator. The evolutionary conservation of Elp1p raises the possibility that it plays a similar role in higher eukaryotes. Mammalian Elp1p is expressed in many tissue types, however the defect in Familial Dysautonomia appears to selectively impact neuronal function. Neurons are amongst the most exquisitely morphologically differentiated cells of the body, highly dependent on polarized exocytosis for their development and function. It is this characteristic of neurons that may make them especially sensitized to the dysregulation of Elp1p function in Familial Dysautonomia. Future studies are necessary to elucidate the details underlying the participation of each Elongator subunit, and to understand the role of mammalian Elongator in the pathophysiology of Familial Dysautonomia.

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Chapter 5. Insights into Elongator's influence on exocytosis

Abstract

Exocytosis is a highly regulated process responsible for cell proliferation and growth. Positive and negative inputs regulate exocytic events in response to signals from other pathways. Earlier work identified a role for the Elongator complex as a negative regulator of exocytosis [1]. Other studies have found that Elongator function influences transcription and translation and recent work indicated that Elongator may regulate exocytosis via a direct regulation of translation [2-4]. We wanted to examine this role and further explore the effect of Elongator function on secretion. Genetic and phenotypic data described here strengthens a role for Elongator function in membrane transport. Suppression analysis of a number of exocytic mutants demonstrates that loss of Elongator has a similar suppression profile to overexpression of the Rab GTPase *SEC4,* the t-SNARE *SEC9,* and to a lesser extent, one of its effector proteins *SRO7*. Phenotypic analysis shows a connection between Swf1p function and Elongator function. Swf1p is a SNARE palmitoyltransferase that is required for growth on 2 mM caffeine at 40^{o}C and, similar to Elongator, this phenotype is suppressed through overexpression of *tQ(UUG)L, tK(UUU)L* tRNA species. Elongator and Swf1p function may provide a link between translation regulation and membrane transport.

Introduction

Intracellular transport of lipids, proteins and other biosynthetic molecules throughout the eukaryotic endomembrane system is essential for cell survival and proliferation. Many regulators of intracellular transport are evolutionarily conserved among eukaryotes. Coat complexes influence vesicle budding, tethering complexes

serve as targeting landmarks, and SNARE proteins trigger vesicle fusion to the target membrane [5]. Rab GTPases are key regulators that target membrane-bound vesicles and organelles to their appropriate destination [5, 6]. Sec4p is an essential exocytic Rab in *S. cerevisae* and serves as a central regulator of transport from the Golgi to the cell surface [7, 8]. Many regulators of exocytosis have been identified, using both genetic and biochemical techniques, where loss of function generally results in a decrease or loss of exocytosis.

Signaling pathways have both positive and negative inputs and our laboratory is interested in understanding the proteins or pathways that elicit a negative regulatory role on exocytosis. A key positive regulator of exocytosis in *S .cerevisae* is the Sec4p guanine nucleotide exchange factor (GEF), Sec2p, that catalyzes the activation step by stimulating GDP for GTP exchange [9]. *ELP1* was identified as an extragenic suppressor of a mutant Sec2p, where deletion suppresses the temperature sensitivity of the *sec2-59* mutant [1]. Elp1p is a component of the Elongator complex [4]. Our initial studies established a role for the Elongator complex, a cytosolic complex with acetyltransferase activity, as a negative regulator of exocytosis.

Reports in the literature demonstrate that Elongator function influences transcription, translation and exocytosis [1, 3, 4]. Elongator was initially identified as a six-subunit complex that copurified with the hyperphosphorylated form of RNA polymerase II (RNAP II) [4, 10, 11]. Conflicting evidence exists, however, that questions if Elongator is a transcription elongation factor as Elongator is a cytosolic resident complex [1, 11-13]. Elongator function also plays a role in translation events as it is required to generate uridine modifications at the wobble position, although the mechanism or stage in the biosynthetic pathway that it catalyzes has yet to be determined [3]. A recent report found that overexpression of two tRNA species could bypass Elongator's negative regulatory role in exocytosis and its effect on

transcription, suggesting that Elongator's direct role is in translation and regulation of exocytosis is a secondary effect [2]. Therefore, we wished to further explore the physiological role of Elongator in exocytosis and the connection to translation.

This report provides an extensive genetic analysis of Elongator's impact on exocytosis. When analyzing exocytic mutants, loss of Elongator function has a similar physiological effect on cell growth to overexpression of *SEC4,* its effector protein *SRO7* and the t-SNARE *SEC9*, proteins that form a ternary complex when Sec4p is GTP-bound [14]. Our data shows that Elongator negatively regulates exocytosis in concert with Elongator-associated proteins Kti11p, Kti12p and Kti13p [15-17]. A broader phenotypic analysis identified genes that phenocopy loss of Elongator function, where approximately 45 nonessential genes are required for cell growth, specifically, on 2 mM caffeine at 40^{o}C. Approximately 40% of these genes have established roles in membrane transport, including organelle acidification, protein sorting and vesicle targeting. A subset of these genes have the caffeine sensitive phenotype suppressed through overexpression of the tRNA species t*Q(UUG)L* and t*K(UUU)L*, including Elongator subunits, *KTI12, KTI13* and the SNARE palymitoyltransferase *SWF1*. This study demonstrates that Elongator function influences membrane transport and may provide a link between translation and intracellular transport regulation.

Materials and Methods

Strains and media

Yeast strains used in this study are listed in Table 5.1. Plasmids used in this study are shown in Table 5.2. YPD (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), SD (0.1% yeast nitrogen base, 2% D-glucose, plus

Table 5.1. Yeast strains used in this study:

Strain Number	Genotype
RCY241	Mat α *ura3-52 leu2-3,112 elp1Δ::URA3*
RCY242	Mat **a** *ura3-52 leu2-3,112 elp1Δ::URA3*
RCY243	Mat α *ura3-52 leu2-3,112 sec1-1*
RCY247	Mat **a** *ura3-52 leu2-3,112 sec3-2*
RCY248	Mat α *ura3-52 leu2-3,112 sec4-8*
RCY250	Mat α *ura3-52 leu2-3,112 sec5-24*
RCY252	Mat α *ura3-52 leu2-3,112 sec6-4*
RCY254	Mat α *ura3-52 leu2-3,112 sec8-9*
RCY255	Mat α *leu2-3,112 sec8-9*
RCY256	Mat **a** *ura3-53 leu2-3,112 sec9-4*
RCY258	Mat α *ura3-52 leu2-3,112 sec10-2*
RCY259	Mat **a** *leu2-3,112 sec10-2*
RCY260	Mat **a** *ura3-52 leu2-3,112 sec15-1*
RCY261	Mat α *leu2-3,112 sec15-1*
RCY271	Mat **a** *ura3-52*
RCY274	Mat α *ura3-52 sec2-59*
RCY421	Mat **a** *ura3-52 sec12-4*
RCY1973a	Mat **a** *ura3-52 leu2-3,112 elp2Δ::KAN^R*
RCY2193	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ypt7ΔKAN^R/ypt7ΔKAN^R*
RCY2195	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 vps21ΔKAN^R /vps21ΔKAN^R*
RCY2219	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ypt52ΔKAN^R/ypt52ΔKAN^R*
RCY2225	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0*
RCY2246	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ypt53ΔKAN^R/ypt53ΔKAN^R*
RCY2867	Mat α *ura3-52 leu2-3,112 elp2Δ::KAN^R*
RCY2872	Mat **a** *ura3-52 leu2-3,112 sec2-59*
RCY2925	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 met15Δ0 elp3Δ::KAN^R*
RCY2965	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 elp1Δ::KAN^R/elp1Δ::KAN^R*
RCY2996	Mat α *ura3-52 leu2-3,112 sec12-4*
RCY3015	Mat **a** *ura3-52 leu2-3,112 his3Δ0 elp3Δ::KAN^R*
RCY3045	Mat **a** *ura3-52 leu2-3,112 sec2-59 elp1Δ::KAN^R*
RCY3254	Mat α *ura3-52 his3Δ0 elp3Δ::KAN^R*
RCY3497	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 tor1Δ::KAN^R*
RCY3541	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 met15Δ0 elp1Δ::KAN^R*
RCY3777	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 met15Δ0 elp2Δ::KAN^R*

Table 5.1 (Continued)

RCY3807	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 elp4Δ::KAN^R*
RCY3815	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 elp6Δ::KAN^R*
RCY4035	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0 his3Δ0/his3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 swf1Δ::KAN^R/swf1Δ::KAN^R*
RCY4036	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 swf1Δ::KAN^R*
RCY4165	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0*
RCY4190a	Mat **a** *ura3-52 trm9Δ::KAN^R*
RCY4248a	Mat **a** *ura3-52 elp5Δ::KAN^R*
RCY4250	Mat **a** *ura3-52 vps21Δ::KAN^R*
RCY4251	Mat **a** *ura3-52 ats1/kti13Δ::KAN^R*
RCY4252	Mat **a** *ura3-52 vps64Δ::KAN^R*
RCY4253	Mat **a** *ura3-52 elp6Δ::KAN^R*
RCY4254a	Mat **a** *ura3-52 kti11Δ::KAN^R*
RCY4255b	Mat **a** *ura3-52 elp4Δ::KAN^R*
RCY4256a	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 elp5Δ::KAN^R*
RCY4268a	Mat **a** *ura3-52 kti12Δ::KAN^R*
RCY4270a	Mat **a** *ura3-52 vma8Δ::KAN^R*
RCY4271a	Mat **a** *ura3-52 cog5Δ::KAN^R*
RCY4272a	Mat **a** *ura3-52 gtr1Δ::KAN^R*
RCY4273a	Mat **a** *ura3-52 chs7Δ::KAN^R*
RCY4276a	Mat **a** *ura3-52 eft2Δ::KAN^R*
RRY673	Mat α *ura3-52 leu2-3,112 sec35-1*

required nutrients), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture) and SPM (0.02% raffinose, 0.3% KOAc) media was used.

Genome-wide analysis to identify genes required for growth on caffeine

The Research Genetics nonessential homozygous diploid knockout collection, where each nonessential gene is systematically knocked out with a kanamycin-resistance cassette, was tested for growth defects on caffeine. Each plate was thawed and used to inoculate liquid YPD + G418 cultures in 96 well deep wells and grown at

Table 5.2. Plasmids used in this study:

Number	Construct	Source
pRC113	*cmyc-SEC2* pRS426	This study
pRC747	*YPT32* pRS426	This study
pRC751	*YPT31* pRS426	This study
pRC1033	*ELP1* pRS315	[1]
pRC1307	*SEC4* pRS425	This study
pRC2076	*ELP1-GFP* pRS316	[1]
pRC2725a	*ELP1D185* pRS316	[1]
pRC2891a	*ELP1* 185 C-terminal residues pRS315	This study
pRC2892b	*ELP1* 185 C-terminal residues pRS425	This study
pR3666a	*tQ(UUG)L, tK(UUU)L* pRS425	This study
3786	*HA-SSO2* pRS316	This study
pB35	*SEC9* pRS426	[24]
pB239	*RHO3* pRS426	[22]
pB496	*KIN1* pRS426	[21]
pB497	*SRO7* pRS426	[24]
pB589	*KIN2* pRS426	[21]
pB1272	$RHO3^{Q74L}$ pRS316	[22]

30°C for 48 hours. This culture was used to inoculate a fresh YPD + G418 culture, which was grown for approximately 15 hours at 30°C. These cultures were used for the analyses. Strains were diluted, through pinning two times into approximately 200 µl of sterile ddH_2O, and then tested for growth on YPD 40°C, YPD+2 mM caffeine 37°C and YPD+2 mM caffeine 40°C plates. Cells were grown for 3 days under the conditions above. Strains that appeared to have the phenotype of interest were tested again to verify the phenotype.

Caffeine sensitive strains were tested for suppression through overexpression of *tQ(UUG)L, tK(UUU)L* (pRC3666a). Three independently created constructs were initially tested against *elpΔ* strains where each resulted in the same phenotype. pRC3666a was used for all subsequent analyses. Strains were transformed and

transformants were selected on minimal media. Transformants (3 to 4 colonies) were spotted on YPD 40°C and YPD+2mM caffeine 40°C and tested for growth for 3 days.

Genetic analysis

A haploid strain carrying the mutation of interest was crossed with a haploid deletion strain. Diploids were isolated using temperature or nutrient selection. If this was not possible, the two haploid strains were crossed on YPD at 25°C for approximately 5 hours. Zygotes were isolated using a micromanipulator on a Zeiss Axiolab microscope and grown on YPD for 2-3 days. Diploids were grown in liquid YPD media to log phase, washed once with sterile water and transferred to SPM media for sporulation. Cells were grown for 5-6 days at room temperature and after sporulation, tetrads were separated into individual spores using a micromanipulator. Spores were grown on YPD 25°C for approximately 3-4 days. Spores were then tested for genotype and phenotype.

Sec4p protein levels in cell lysates

Whole cell lysates were made in wildtype and *elp1Δ* cells (RCY239 and RCY242 respectively). Cells were lysed using glass beads in TAZ buffer (10mM Tris pH7.5, 10mM azide) with protease inhibitors (1mM phenylmethylsulphonyl fluoride, 10ug/ml pepstatin, 1mM benzamidine). Sample buffer was added, samples were incubated at 60°C for 10 minutes. Samples were loaded on a 12% SDS-PAGE gel. Contents of the gel were transferred to polyvinylidene fluoride (PVDF) for 3 hours at 200mA. Sec4p protein levels were detected using an αSec4p polyclonal chicken antibody at 1:300. Elp1p protein levels were detected using an αElp1p polyclonal chicken antibody at 1:1000. The Western blot was stripped and probed with α10D7 (αVac ATPase) for a loading control. The Western blots were developed with

chemiluminscence (CDP Star) using a Fuji Film LAS-3000 imager and data was collected using Image Reader LAS-3000 software.

Subcellular fractionation

Wildtype, *elp1Δ*, and *swf1Δ* cells (RCY2225, 2965 and 3045, respectively) were transformed with *HA-SSO2* and vector only or *tQ(UUG)L, tK(UUU)L*, selected for on minimal media and transformants were grown to mid log phase. 5 OD units (ODU) were lysed into 150 μl of PBS, 0.2M sorbitol, protease inhibitors (1mM phenylmethylsulphonyl fluoride, 10ug/ml pepstatin, 1mM benzamidine), and 2mM azide. 400 μl PBS+0.2M sorbiol was added and mixed and lysates were cleared using centrifugation at 500xg for 5 minutes at 4^{o}C. 200ul of cleared lysates were centrifuged at 100,000xg at 4^{o}C for 20 minutes. The supernatant (S100) fraction was collected and the pellet was rinsed with 100ul of PBS+0.2M Sorbitol. The pellet was resuspended with 200ul PBS+3% Triton-X 100 (P100). Sample buffer was added to a final concentration of 0.75% sucrose, 0.75% SDS, Tris pH6.8 and 2-mercaptoethanol. 5.5ul of each sample was loaded per lane for approximately 0.51ODU per lane on a 10% SDS PAGE gel. Contents on the gel were transferred to polyvinylidene fluoride (PVDF) and HA-Sso2p was detected using and HA α-chicken antibody. Alkaline phosphatate-conjugated goat anti-chicken secondary antibody was then used to probe and HA-Sso2p was developed with chemiluminscence (CDP Star) using a Fuji Film LAS-3000 imager and data was collected using Image Reader LAS-3000 software.

Results

All six Elongator subunits are required for negative regulation of exocytosis

Previous work in the laboratory established that Elongator subunits, *ELP1, ELP2,* and *ELP3,* negatively regulate exocytosis [1]. The holo-Elongator complex

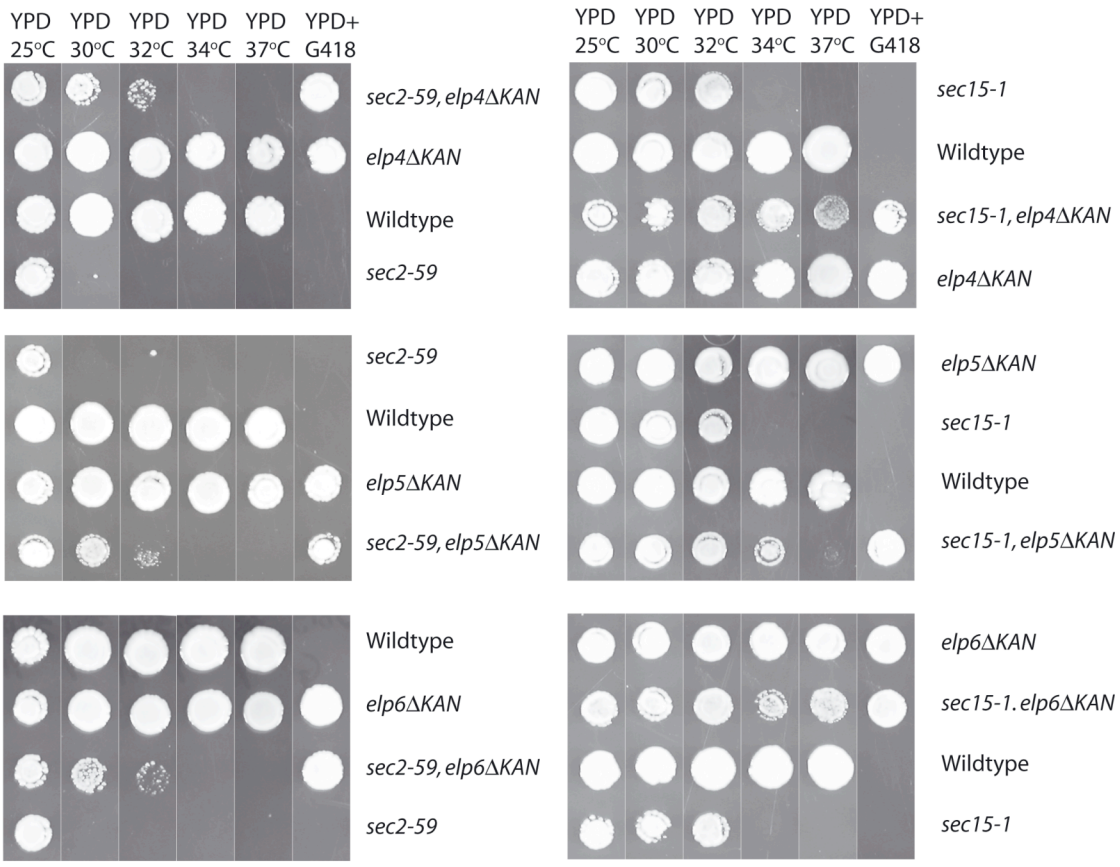


Figure 5.1. ***ELP4, ELP5, ELP6* are required for Elongator negative regulation of exocytosis**. Strains containing *elp4Δ*, *elp5Δ*, or *elp6Δ* were crossed with a mutant strain containing *sec2-59* (left column) or *sec15-1* (right column). Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

consists of two discrete subcomplexes, Elp1p-Elp3p and Elp4p-Elp6p, which can be separated biochemically [10, 12]. We were interested in determining if the holo-Elongator complex is required for negative regulation of exocytosis or if only subunits Elp1p-Elp3p function in this process. Our analysis found that *ELP4, ELP5* and *ELP6* also influence exocytosis (Figure 5.1). Deletion of either gene suppresses the temperature sensitivity of the mutant Rab GEF *sec2-59* and a mutant Exocyst subunit *sec15-1*. The Exocyst complex is proposed to function as a tethering complex at the plasma membrane that targets post-Golgi vesicles where subunit Sec15p is a direct

effector of Sec4p to facilitate tethering [18]. Taken together with previous data, all six subunits are required to negatively regulate exocytosis. This is consistent with previous findings where deletion of any Elongator subunit gives rise to similar phenotypes [3, 4, 10, 11, 17].

Genetic Analysis of Elongator's effect on late-acting secretory mutants

While our data implicated the Elongator Complex as a negative regulator of exocytosis, the particular nature of the regulation was unclear [1]. Mutations of the Sec4p guanine nucleotide exchange factor, *SEC2*, are suppressed through loss of Elongator function. The overall effect of Elongator function on the post-Golgi secretory pathway was unknown and we reasoned determining this may give insights into its role. We took advantage of temperature sensitive mutants of many key regulators of post-Golgi transport to assess Elongator's functional impact on exocytic events. Temperature sensitive mutants of the Rab GTPase Sec4p (*sec4-8*), Exocyst subunits (*sec3-2, sec5-24, sec6-4, sec8-9, sec10-2, sec15-1*), the exocytic t-SNARE Sec9p (*sec9-4*), and the exocytic SM protein involved in stimulating vesicle fusion Sec1p (*sec1-1*), were crossed with deletion strains of either *ELP1*, *ELP2* or *ELP3*. In addition, a mutant in the Golgi tethering complex (COG Complex) Sec35p (*sec35-1*) and ER Arf GTPase exchange factor Sec12p (*sec12-1)* were used to identify genetic interactions between mutants earlier in the secretory pathway. As stated before, deletion of any Elongator subunit generally gives rise to a similar phenotype. Therefore we felt assessing the genetic interactions of three Elongator subunits would give a general insight into the effect of holo-Elongator function on exocytosis.

Figure 5.2a shows the genetic interactions between Elongator subunits *ELP1, ELP2* and *ELP3* and the late-acting secretory mutants tested. It is interesting to note that the overall suppression profile of loss of Elongator function is similar to

	elp1Δ	*elp2Δ*	*elp3Δ*	*SEC4 overexpression*
sec1-1	++	++	++	+
sec2-59	++	++	++	++++
sec3-2	++	++	-	++
sec4-8	++	++	++	++++
sec5-24	+	++	-	++
sec6-4	+/-	+/-	+/-	-
sec8-9	++	++	++	++++
sec9-4	-	-	-	-
sec10-2	-	-	-	++
sec15-1	++	++	++	++++
sec35-1	-	ND	ND	-
sec12-4	-	ND	ND	-

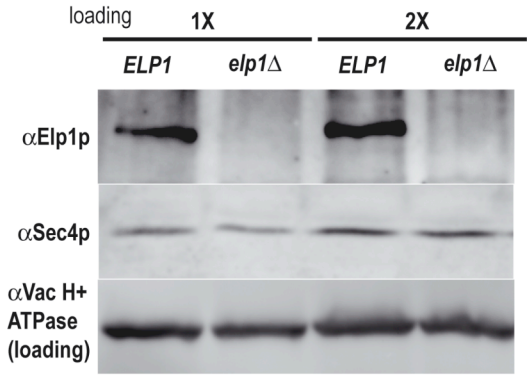


Figure 5.2. Genetic analysis of Elongator deletions with secretory mutants. A) A summary of the genetic interactions between Elongator deletion and secretory mutants. (++++) indicates suppresses back to wildtype, (+++) very strong suppression, (++) strong suppression, (+) suppression, (+/-) weak suppression, (-) no genetic interaction. B) Western blot analysis was used to measure proteins levels in whole cell lysates in *ELP1* and *elp1Δ* cells. αElp1p antibody was used to measure Elp1p protein levels, αSec4p antibody was used to measure Sec4p protein levels, and αVac H^+ ATPase was used as a loading control. Figure shows two levels of protein loading.

overexpression of *SEC4* (Figure 5.2a, column 4). The similarity between loss of Elongator function and overexpression of *SEC4* is not a result, however, of merely raising the Sec4 protein levels as the protein levels remain constant in *ELP1* and *elp1Δ* cells (Figure 5.2b). The genetic interaction profile is not similar to overexpression of the exchange factor *SEC2*, as we found overexpression of *myc-SEC2* only suppressed *sec2-59* and weakly suppressed *sec15-1* mutants (data not shown). This data suggests that Elongator may influence the function of the Rab GTPase that regulates post-Golgi vesicular transport, Sec4p.

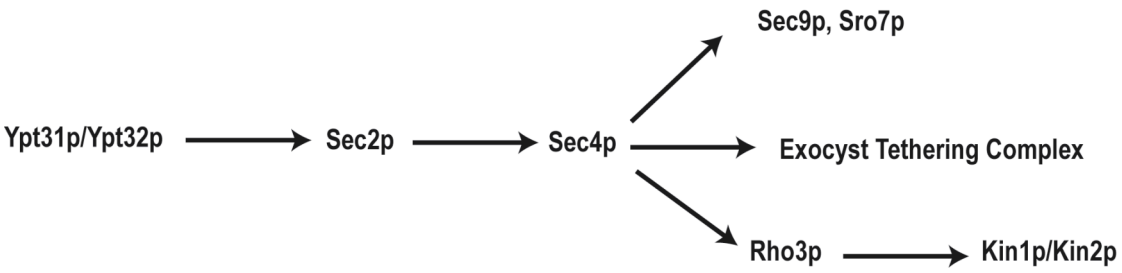


Figure 5.3. Positive regulators of exocytosis.

Many proteins functioning upstream and downstream of Sec4p have been identified, including the Golgi Rabs Ypt31p and Ypt32p, the t-SNARE Sec9p, the Sec4p effector Sro7p, Rho3p, and two kinases involved in polarity determination Kin1p and Kin2p (Figure 5.3) [19-24]. These proteins function to promote exocytosis and overexpression of each suppresses a subset of secretory mutants. We reasoned that comparing the suppression profiles of overexpression of each positive regulator to loss of Elongator function might provide insights into the stage in the exocytic pathway that Elongator regulates.

Overall, the suppression profile of loss of Elongator function was similar to overexpression of the Rab *SEC4* and also overexpression of the Sec4p effector protein *SRO7* and the t-SNARE *SEC9* (Table 5.3). Sec4p interacts with Sro7p when GTP-bound and these proteins form a ternary complex with Sec9p [14]. Two examples of the analysis used to generate the suppression profiles are shown in Figure 5.4a and 5.4b, assessing the suppression of *sec1-1* and *sec15-1* mutants. Deletion of an Elongator subunit, overexpression of *SEC4* and overexpression of *SEC9* and *SRO7* suppress a number of late-acting secretory mutants including *sec2-59, sec4-8, sec1-1* (not *SRO7*) and multiple Exocyst mutants. Expression of constitutively active *RHO3*

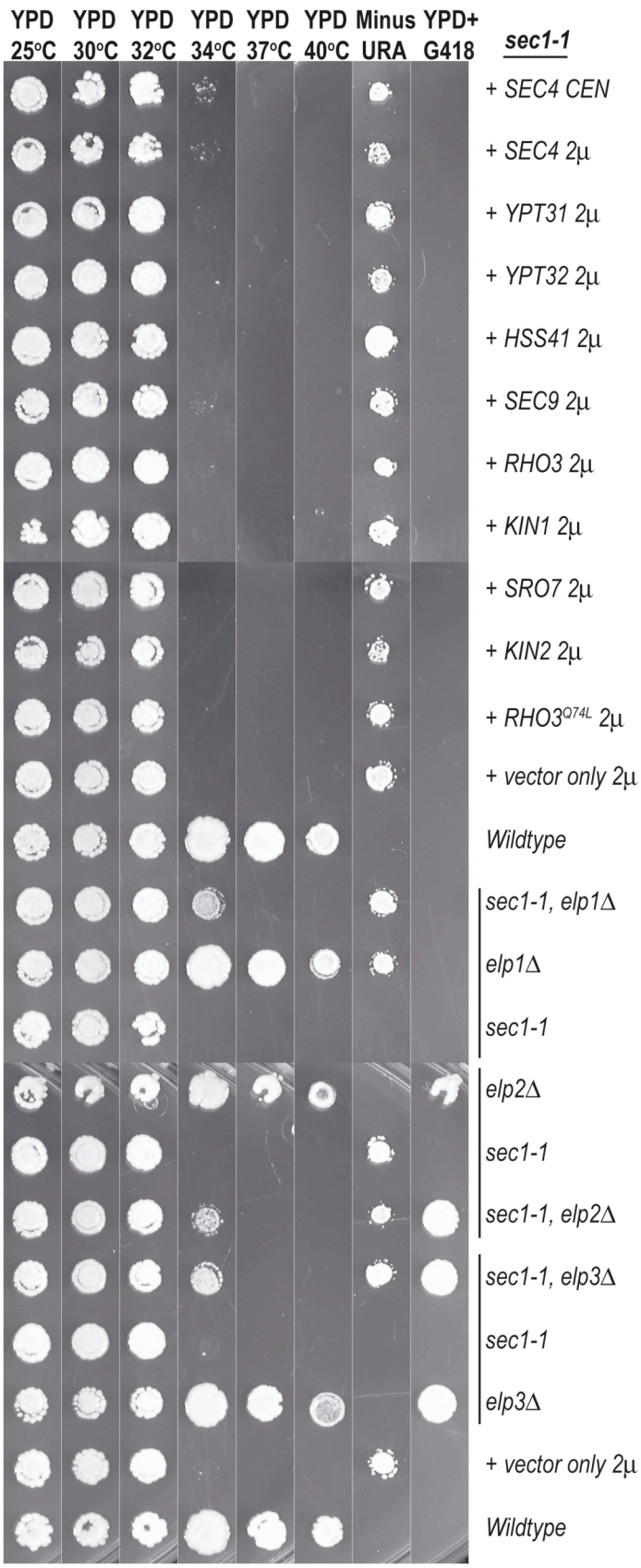
Table 5.3. Suppression of exocytic mutants through deletion of Elongator subunits or overexpression of regulators of exocytosis. (++++) indicates suppresses back to wildtype, (+++) very strong suppression, (++) strong suppression, (+) suppression, (+/-) weak suppression, (-) no genetic interaction.

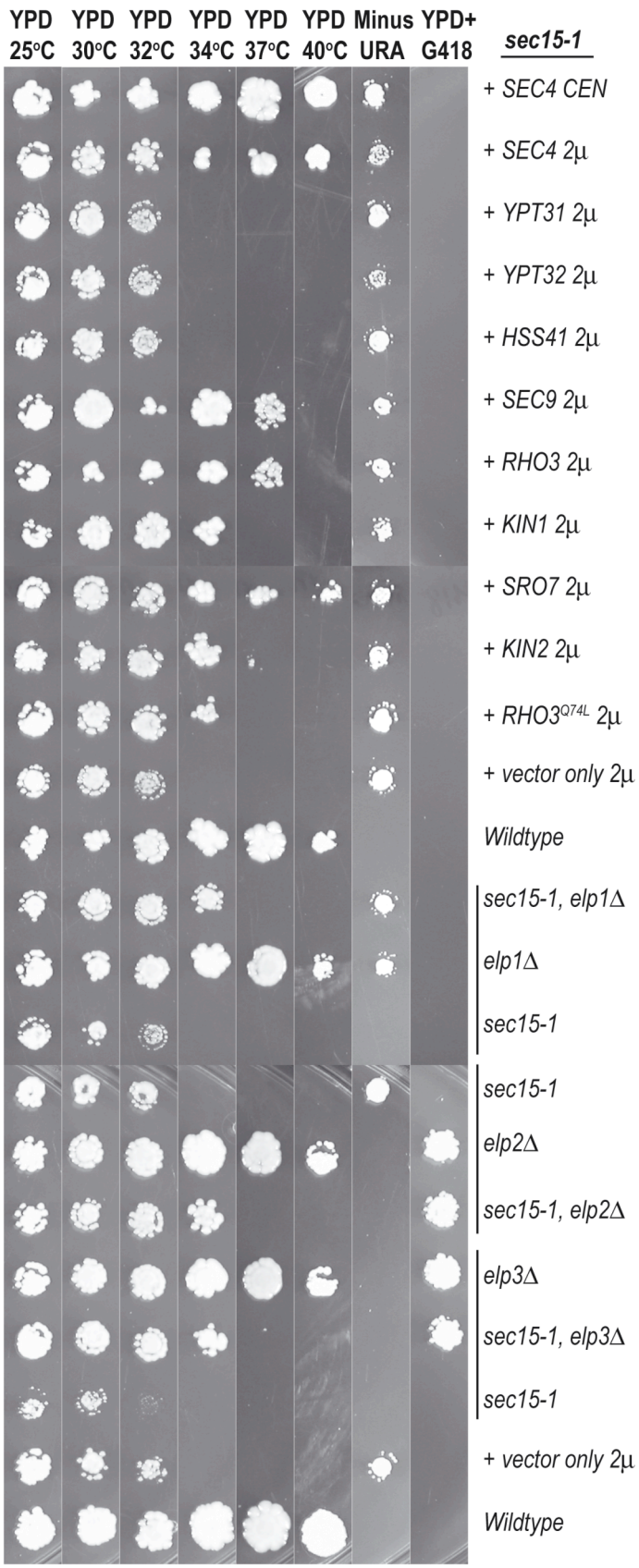
	DELETION			OVEREXPRESSION								
	elp1	*elp2*	*elp3*	*SEC4*	*SEC9*	*SRO7*	*RHO3*	*RHO3*^{Q74L}	*KIN1*	*KIN2*	*YPT31*	*YPT32*
sec1-1	++	++	++	+	+/-	-	-	-	-	-	-	-
sec2-59	++	++	++	++++	++	++	++	++	++	++	-	++++
sec3-2	++	++	-	++	++	++++	-	-	-	-	-	-
sec4-8	++	++	++	++++	++	+	+	-	++	++	-	++
sec5-24	+	++	-	++	-	-	-	-	-	-	+	-
sec6-4	+/-	+/-	+/-	-	-	-	-	-	-	-	-	-
sec8-9	++	++	++	++++	++	++	-	-	-	-	-	-
sec9-4	-	-	-	-	++++	-	-	-	-	-	-	-
sec10-2	-	-	-	++	-	-	-	-	-	-	-	-
sec15-1	++	++	++	++++	++	++++	+++	++	++	++	-	-
sec35-1	-	ND	ND	-	-	-	-	-	-	-	-	-
sec12-4	-	ND	ND	-	-	-	-	-	-	-	-	-

(*RHO3*^{Q74L}), overexpression of *RHO3* and the kinases *KIN1* and *KIN2* have a similar suppression profile in that they specifically suppress *sec2-59, sec4-8* and *sec15-1* mutants, but do not suppress other Exocyst mutants or a Sec1p mutant, suggesting Elongator does not function at this stage in exocytosis. Also, overexpression of the Golgi Rabs, *YPT31* and *YPT32*, that function upstream of Sec4p and recruits Sec2p to post-Golgi vesicles do not have a broad suppression effect on late-acting secretory mutants, suggesting Elongator does not function at this stage either [20].

Deletion of *ELP* subunits does not suppress *sec9-4*, however the overall profile is similar to overexpression of *SEC9*. This suggests Elongator may function upstream of Sec9p. Furthermore, the genetic profiles suggest a physiologic link between Elongator function and the ability of Sec4p to act on the t-SNARE Sec9p and Sro7p.

Figure 5.4. Suppression analysis of *sec1-1* and *sec15-1.* A) *sec1-1* cells were transformed with the indicated plasmid and grown at 25^{o}C. Transformants were grown on the indicated media and temperature to test for grown. On the same plate, strains containing *sec1-1* with an Elongator deletion was tested for growth. Isogenic strains (*elp*Δ, *sec1-1, elp1Δ sec1-1*) from a single tetrad are grouped by a line. Cells grown for three days. B) *sec15-1* cells were transformed with the indicated plasmid and grown at 25^{o}C. Transformants were grown on the indicated media and temperature to test for grown. On the same plate, strains containing *sec15-1* with an Elongator deletion was tested for growth. Isogenic strains (*elp*Δ, *sec15-1, elp1Δ sec15-1*) from a single tetrad are grouped by a line. Cells grown for three days.


YPD 25°C
YPD 30°C
YPD 32°C
YPD 34°C
YPD 37°C
YPD 40°C
Minus URA
YPD+ G418
sec1-1
+ SEC4 CEN
+ SEC4 2μ
+ YPT31 2μ
+ YPT32 2μ
+ HSS41 2μ
+ SEC9 2μ
+ RHO3 2μ
+ KIN1 2μ
+ SRO7 2μ
+ KIN2 2μ
+ RHO3Q74L 2μ
+ vector only 2μ
Wildtype
sec1-1, elp1Δ
elp1Δ
sec1-1
elp2Δ
sec1-1
sec1-1, elp2Δ
sec1-1, elp3Δ
sec1-1
elp3Δ
+ vector only 2μ
Wildtype


YPD 25°C
YPD 30°C
YPD 32°C
YPD 34°C
YPD 37°C
YPD 40°C
Minus URA
YPD+ G418
sec15-1
+ SEC4 CEN
+ SEC4 2μ
+ YPT31 2μ
+ YPT32 2μ
+ HSS41 2μ
+ SEC9 2μ
+ RHO3 2μ
+ KIN1 2μ
+ SRO7 2μ
+ KIN2 2μ
+ RHO3Q74L 2μ
+ vector only 2μ
Wildtype
sec15-1, elp1Δ
elp1Δ
sec15-1
sec15-1
elp2Δ
sec15-1, elp2Δ
elp3Δ
sec15-1, elp3Δ
sec15-1
+ vector only 2μ
Wildtype

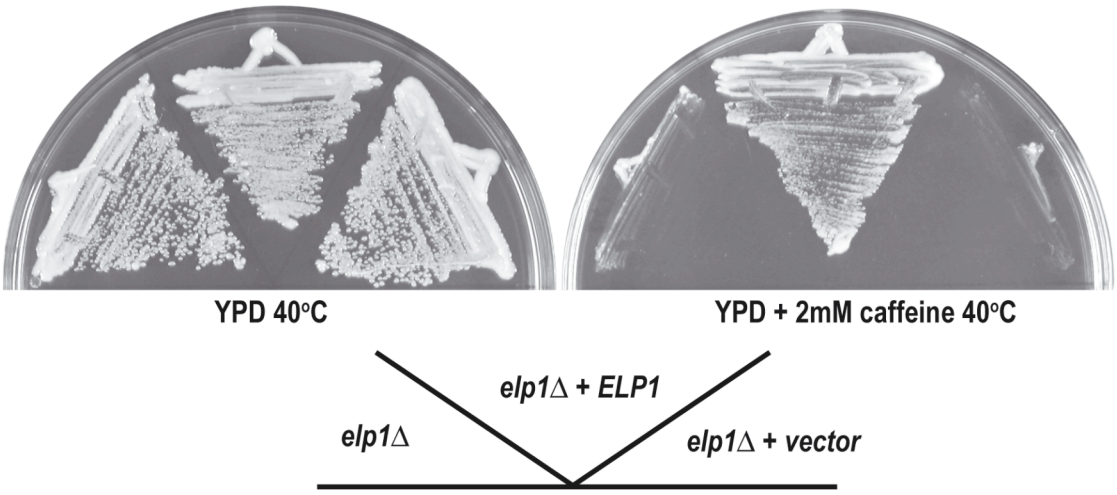


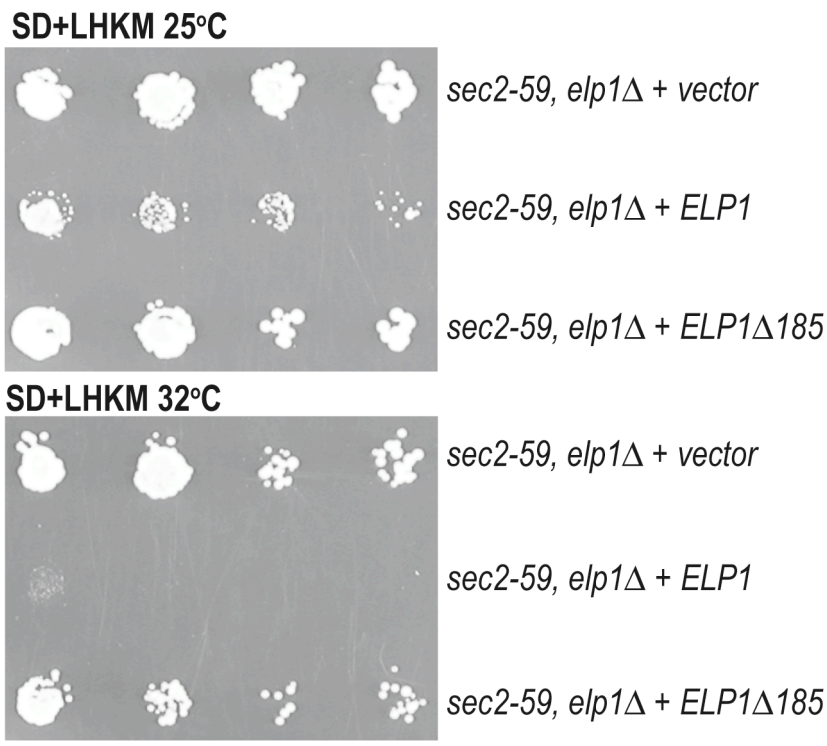
Figure 5.5. Caffeine sensitivity caused by loss of Elongator function. A strain containing *elp1Δ* was tested for growth on YPD 40°C, YPD+2mM caffeine 37°C (not shown) and YPD+2mM caffeine 40°C with no insert, vector only, or *ELP1* plasmid. Cells grown for three days.

Identification of genes that phenocopy loss of Elongator function

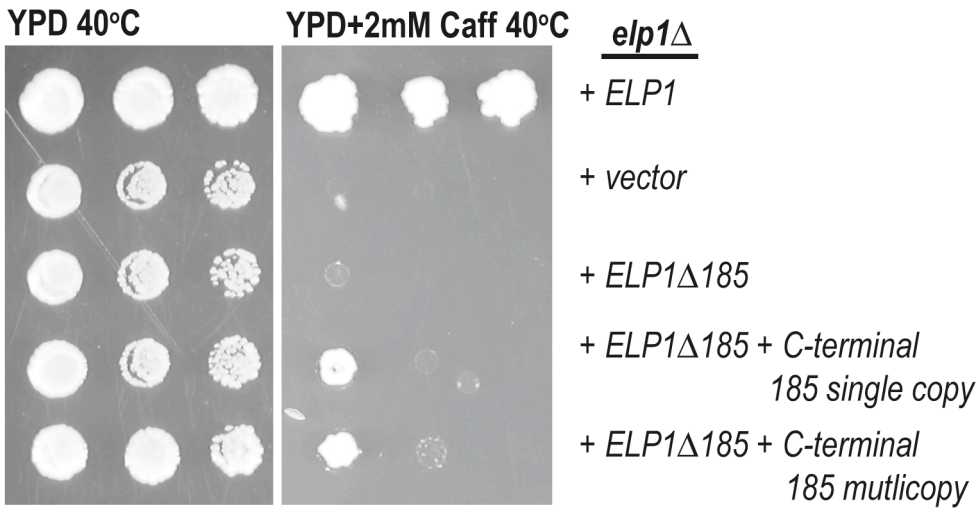
The suppression analysis focused on the effect of Elongator function on mutants that have known roles in post-Golgi vesicular transport. An unbiased approach was taken to get a broader idea of Elongator complex function and other proteins that may be involved in the same pathway. Loss of Elongator function causes sensitivity to 2mM caffeine when grown on YPD media at 40°C (Figure 5.5). For example, *elp1Δ* cells are viable on YPD at 40°C and also YPD+2mM caffeine at 37°C, however, specifically sensitive for growth on YPD+2mM caffeine at 40°C. We found through mutational analysis that Elp1p requires its C-terminal 185 residues for growth on 2mM caffeine at 40°C and also to negatively regulate exocytosis (Figure 5.6a, b). Supplementing an *elp1Δ* strain with a truncated *ELP1* fails to compliment a full-length protein for growth on caffeine and for restoring thermosensitivity to *sec2-59 elp1Δ* cells. Interestingly, addition of the C-terminal 185 residues in trans (at single copy and overexpressed levels) can partially compliment wildtype *ELP1* for growth on caffeine

Figure 5.6. Elp1p C-terminal 185 residues for functionality. A) Vector only, wildtype *ELP1* and *ELP1Δ185* constructs were transformed into a *sec2-59 elpΔKAN* strain. Transformants were tested for growth on minimal media, supplemented with the required nutrients, at 25°C and 32°C. Functionality in exocytosis can be assessed as restoration of temperature sensitivity at 32°C. Growth is tested using four different cell dilutions for three days. B) Vector only, wildtype *ELP1*, *ELP1Δ185, ELP1Δ185* + C-terminal 185 residues single copy, and *ELP1Δ185* + C-terminal 185 residues multi copy constructs were transformed into *elp1Δ* cells. Functionality can be assessed by growth on YPD + 2mM caffeine at 40°C. Growth is tested using three different cell dilutions for three days.

A)


SD+LHKM 25°C
sec2-59, elp1Δ + vector
sec2-59, elp1Δ + ELP1
sec2-59, elp1Δ + ELP1Δ185
SD+LHKM 32°C
sec2-59, elp1Δ + vector
sec2-59, elp1Δ + ELP1
sec2-59, elp1Δ + ELP1Δ185

B)


YPD 40°C
YPD+2mM Caff 40°C
elp1Δ
+ ELP1
+ vector
+ ELP1Δ185
+ ELP1Δ185 + C-terminal 185 single copy
+ ELP1Δ185 + C-terminal 185 mutlicopy

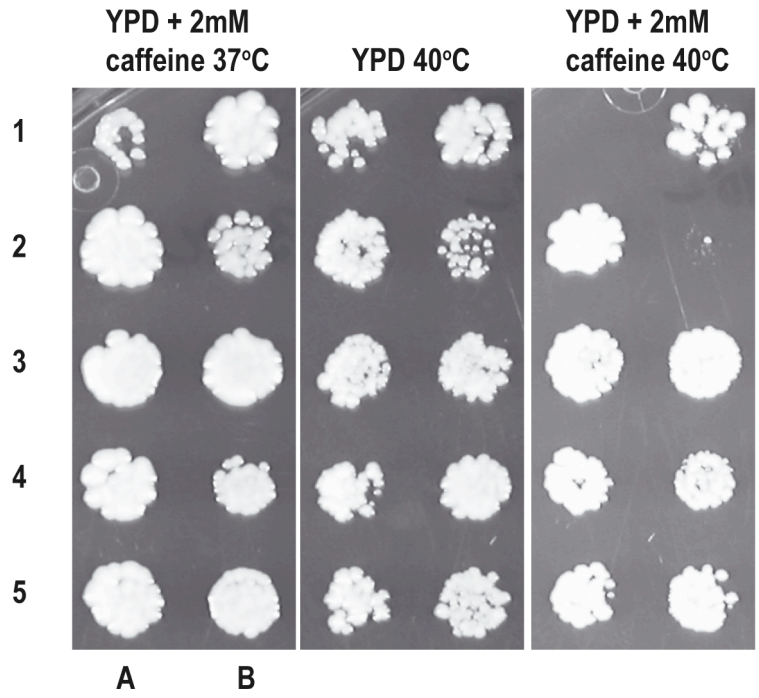


Figure 5.7. Example of caffeine sensitive strain. An example result from testing strains for growth on YPD 40°C, YPD+2mM caffeine 37°C and YPD+2mM caffeine 40°C. Cells are grown for three days.

(Figure 5.6b). The caffeine sensitivity at high temperatures that is observed in these cells is a very specific phenotype that we reasoned could be utilized to screen for other genes that display the same phenotype. We wished to identify other genes that are required for growth on 2mM caffeine at 40°C as this might lead to a better understanding of Elongator's *in vivo* role and physiological impact.

The *Saccharomyces* non-essential homozygous diploid knockout collection was screened to identify deletion strains that phenocopy loss of Elongator function. Strains were grown overnight in liquid YPD media containing G418 and spotted on YPD 40°C, YPD+2mM caffeine 37°C and YPD+2mM caffeine 40°C. Cells were grown for 3 days and strains were analyzed for growth. Strains that appeared to display the phenotype of interest were subject to an additional round of testing for

growth defects under the conditions listed above. An example of a typical analysis is shown in Figure 5.7, where strains A1 and B2 are sensitive for growth on 2mM caffeine at 40^{o}C.

At least 1% of the nonessential genome displays this phenotype, including genes that have been implicated in biosynthetic processes, cytoskeleton organization, transcription, cell cycle regulation, intracellular transport and organelle structural integrity (Table 5.4). Three Elongator subunits were uncovered, *ELP2, ELP4, ELP6*, and the Elongator associated protein *KTI12* [17]. *ATS1/KTI13* and *CHS7* share other phenotypes to Elongator, including resistance to zymocin toxin [16, 25]. *ATS1/KTI13* and *CHS7* also share a caffeine sensitivity phenotype with Elongator and further verifies the screen design. The fact that all six subunits were not identified indicates that some genes that display this phenotype were missed in our screen, which is common among large-scale screens. Nevertheless, our analysis gained valuable insights into additional pathways where disruption phenocopies loss of Elongator function.

Elp3p contains a putative SAM (*S-a*denosyl *m*ethionine) radical domain that can perform a variety of radical reactions where SAM is cleaved for methyl transfer [26]. Genes that may be involved in SAM biosynthesis were identified as caffeine sensitive. *ADO1* and *ADK1* encode for adenosine kinases that are required for cells to utilize SAM as a purine source [27, 28]. Erg4p is involved in the final step of ergosterol biosynthesis and loss of function was recently shown to lead to elevated SAM levels in cells [29, 30]. Deletion of *ADO1, ADK1* or *ERG4* results in sensitivity to caffeine. These genes may be involved in regulating cellular SAM concentration, and therefore, regulating substrate levels in the cell for one of Elongator's proposed catalytic activities.

Table 5.4. Genes that are required for growth on YPD+2mM caffeine 40°C, but not required for growth on YPD 40°C or YPD+2mM caffeine 37°C. Functional groups are derived from the Yeast Genome database at www.yeastgenome.org

	ORF Name	ORF Number	SGD Function/Process
cytoskeleton	*ATS1/KTI13*	YAL020C	microtubule organization and biogeneisis
	ADO1	YJR105W	adenosine kinase activity
	TPM1	YNL079C	tropomyosin major isoform, stabilizes actin cables
cell cycle	*TOR1*	YJR066W	target of rapamycin, kinase that regulates cell growth in response to nutrients
	DBF2	YGR092W	kinase involved in mitotic exit
biosynthesis	*REG1*	YDR028C	protein phosphatase type 1 regulator subunit
	ADK1	YDR226W	adenylate kinase, required for purine metabolism
	ERG4	YGL012W	C-24(28) sterol reductase, catalyzes the final step in ergosterol biosnythesis
	PRS3	YHL011C	involved in 5-phospho-ribosyl-1(alpha)-pyrophosphate synthesis and nucleotide synthesis
uncharacterized	*BUD30*	YDL151C	unlikely to encode protein
	hyp orf	YER186C	hypothetical protein
	hyp orf	YOR309C	hypothetical protein
	unchar orf	YNL080C	uncharacterized protein, predicted transmembrane protein
organelle structure	*SMI1*	YGR229C	involved in the regulation of cell wall synthesis
	SPO7	YAL009W	required for normal nuclear envelope morphology
	NEM1	YHR004C	required for normal nuclear envelope morphology
transcription	*CCR4*	YAL021C	component of the CCF4-NOT complex, polyA tail shortening, implicated in many functions
	TUP1	YCR084C	involved in the general repression of transcription
miscellaneous	*ELP2*	YGR200C	elongator complex subunit
	ELP4	YPL101W	elongator complex subunit
	ELP6	YMR312W	elongator complex subunit
	KTI12	YKL110C	associated with elongator complex
metabolism	*ATP15*	YPL271W	subunit of F1F0 ATP synthase
membrane transport	*PMT2*	YAL023C	protein O-mannosyltransferase at the ER
	SWF1	YDR126W	SNARE palmitoyltransferase
	VPS21	YOR089C	endocytic Rab GTPase
	VAC1/VPS19	YDR323C	facilitates vacuolar protein sorting, Vps21p effector
	PIB2	YGL023C	binds PI-3,P, similar to Fab1 and Vps27, involved in vesicle transport
	VPS64/FAR9	YDR200C	required for cytoplasm to vacuole protein targeting
	CHS7	YHR142W	regulates Chs3p export from ER to regulate chitin biosynthesis
	SPC2	YML055W	subunit of signal peptidase complex with Spc1, Spc3, Sec11
	COG5	YNL051W	subunit of the conserved oligomeric complex, Golgi tethering complex
	COG7	YGL055C	subunit of the conserved oligomeric complex, Golgi tethering complex
	COG8	YML071C	subunit of the conserved oligomeric complex, Golgi tethering complex
	PPA1/VMA16	YHR026W	subunit c" of the vacuolar ATPase, functions in acidification of the vacuole
	VMA13	YPR036W	subunit H of the vacuolar ATPase, acidification throughout the endomembrane system
	VMA8	YEL051W	subunit D of the vacuolar ATPase, acidification throughout the endomembrane system
	VPH2/VMA12	YKL119C	found in the ER, functions in vacuolar ATPase assembly
	VPS3	YDR495C	required for sorting and processing of soluble vacuolar proteins
	GTR1	YML121W	cytoplasmic GTPase subunit of GSE complex, involved in endoctic sorting
	GTR2	YGR163W	cytoplasmic GTPase subunit of GSE complex, involved in endoctic sorting

Approximately 40% of the genes required for growth on 2mM caffeine at 40^{o}C have a function associated with various aspects of membrane transport. *PMT2* and *SWF1* encode proteins that posttranslationally modify target proteins and are required for growth under this condition [31, 32]. Pmt2p is an O-mannosyltransferase that glycosylates proteins at the ER that are destined for transport to the Golgi apparatus [31]. Swf1p is a palmitoyltransferase whose *in vivo* targets are SNARE proteins [32, 33]. Swf1p-dependent palmitoylation influences SNARE stability, where it inhibits ubiquitination and degradation [32].

Tpm1p is one of two tropomyosin isoforms in yeast that stabilizes actin cables and has direct roles in vesicular transport [34, 35]. Disruption of *TPM1* leads to a disappearance of actin cables and we found also causes sensitivity to growth on 2mM caffeine at 40^{o}C [36].

VPS21 encodes an endocytic Rab GTPase that regulates transport of molecules into the prevacuolar compartment (PVC), via early endosomes or the Golgi [37]. Vac1p/Vps19p binds phosphatidylinositol 3-phosphate (PI 3-P) and regulates vacuolar protein sorting. In addition, Vac1p is a Vps21p effector that preferentially binds Vps21p in its GTP-bound state [38]. Loss of either protein results in caffeine sensitivity, indicating Vps21p-dependent membrane transport is required for growth under these conditions.

Transport through the endocytic pathway is regulated by four nonessential Rab GTPases: Vps21p, Ypt52p, Ypt53p and Ypt7p. Caffeine has been shown to inhibit endocytosis in *Dictyostelium discoidium*, however its mode of action in *S. cerevisae* is unknown [39]. Therefore it is intriguing that the endocytic Rab Vps21p and its effector protein were identified in this screen, in addition to other genes that encode proteins that have a role in endocytic events (*PIB2, VPS64, VPS3*, *VMA12* and three

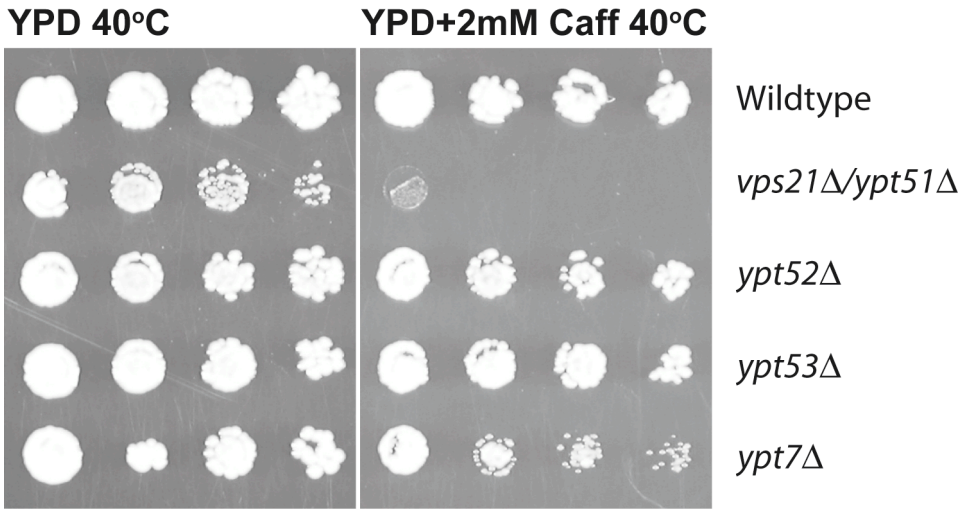


Figure 5.8. Caffeine sensitivity caused by loss of endocytic Rabs. A diploid strain containing either *vps21Δ, ypt52Δ, ypt53Δ,* or *ypt7Δ* was tested for growth on YPD 40°C and YPD+2mM caffeine 40°C for three days.

vacuolar ATPase subunits). To determine whether only the Vps21p-dependent stages of transport display growth defects on caffeine or if all stages of endocytosis are effected, a deletion of each endocytic Rab GTPase was tested for growth on YPD 40°C and YPD+2mM caffeine 40°C. Figure 5.8 shows that only *vps21*Δ cells display growth inhibition on YPD+2mM caffeine, indicating this is the only endocytic stage of transport essential for growth on 2mM caffeine at 40°C. Pulse-chase analysis using the endocytic dye FM4-64 confirmed that 2mM caffeine does not inhibit all endocytic events, in cells pretreated with caffeine for up to 5 hours prior to analysis (data not shown).

The vacuolar ATPase acidifies organelles throughout the endomembrane system. An acidification gradient exists that plays an important role in protein sorting, organelle identity, and protein degradation through different compartments. Deletion of three vacuolar ATPase subunits, *VMA13, VMA16,* and *VMA8*, results in caffeine sensitivity [40, 41]. Also, deletion of *VMA12*, which assembles the vacuolar ATPase

at the ER, causes caffeine sensitivity [42]. Not every vacuolar ATPase subunit was identified, but multiple proteins involved in regulation of organelle pH are required for growth in the presence of 2mM caffeine at 40^{o}C, suggesting that regulation of organelle pH, at some level, supports growth under this condition.

Two small GTPases, *GTR1* and *GTR2*, that are essential for proper sorting of the amino acid permease Gap1p out of endosomes, are also required for growth on 2mM caffeine at 40^{o}C [43]. These two proteins form a complex (the GSE complex) with Gse1p, Gse2p and Ltv1p that facilitates protein sorting in endosomes. The authors of this study hypothesize these five proteins may form an endosomal coat complex where Gtr1p and Gtr2p function as GTPases to recruit coat complexes, similar to Arf1p and Sar1p [43, 44].

The COG (*c*onserved *o*ligomeric *G*olgi) complex is an evolutionarily conserved tethering complex at the Golgi. It is composed of eight subunits, four essential (*COG1, COG2, COG3,* and *COG4*) and four nonessential (*COG5, COG6, COG7,* and *COG8*). Structural and biochemical studies have suggested that the complex adopts a bi-lobed architecture, where one lobe consists of the essential subunits, Cog1p-3p, the second lobe consists of the nonessential subunits, Cog5p-8p, and Cog4p serves as a bridge between the two lobes [45, 46]. Three nonessential COG subunits, *COG5, COG7* and *COG8* (all located in COG lobe B) are essential for growth on 2mM caffeine at 40^{o}C.

Multiple components of different complexes were identified in this screen in addition to Elongator. This gives confidence in suggesting that an aspect of COG complex function, GSE complex function, and vacuolar ATPase function are essential for growth on caffeine at high temperatures. Another complex required for growth under these conditions is the complex formed by Spo7p and Nem1p. These proteins are required to properly shape the nuclear envelope [47].

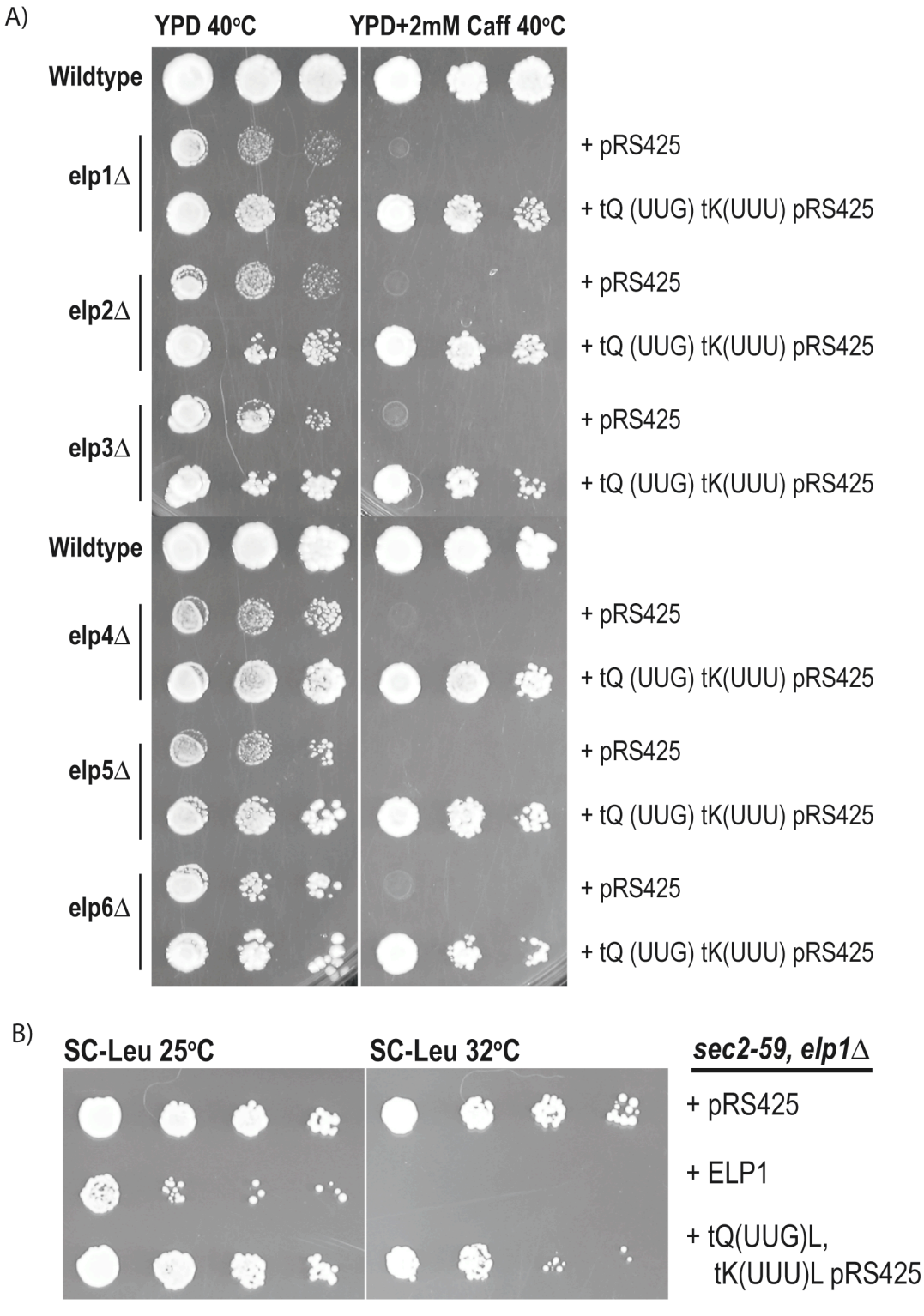
Tor1p, the target of rapamycin, is a kinase that regulates cell growth and proliferation in response to nutrients [48]. Loss of *TOR1*, but not *TOR2*, results in sensitivity to caffeine. Interestingly, loss of Elongator function gives rise to hypersensitivity towards growth in the presence of rapamycin, suggesting Elongator positively functions in the Tor1p signaling pathway (data not shown).

Elongator's role in translation regulation

The Tor1p pathway has been shown to regulate cell proliferation at the translational level [48]. The Elongator complex also functions in an aspect of translation, as it provides function in a step in the biosynthetic pathway to generate a modified uridine at the wobble position [3]. Proteins directly involved in translation were not identified in the caffeine sensitivity screen; however, *SPC2,* which encodes for a signal peptidase complex (SPC) subunit is required for growth on caffeine at high temperatures. The SPC cleaves off the signal peptides on nascent proteins as they are translated and pass through the ER translocation complex [49, 50]. Spc2p is required for full SPC activity and associates with subunits of the translocon [49].

A recent report identified two tRNA species that bypass Elongator's role in exocytosis, suggesting that Elongator influences secretion independently through its role in translation [2]. In this study, the authors found that overexpression of the tRNA species t*Q(UUG)L* and t*K(UUU)L* can bypass the caffeine sensitivity caused by loss of Elongator function. In addition, overexpression restores the thermosensitivity of a *sec2-59 elp1Δ* strain. We found that overexpression of these two tRNA species does suppress the caffeine sensitivity of loss of each Elongator subunit (Figure 5.9a). To date, we have been unable to restore the thermosensitivity of a *sec2-59 elp1Δ* strain through overexpression of t*Q(UUG)L* and t*K(UUU)L* using three independently created constructs that suppress the caffeine sensitivity (Figure 5.9b). We have yet to

Figure 5.9. Suppression of Elongator caffeine sensitivity through overexpression of two tRNA species. A) Cells with a deletion of each Elongator subunit was transformed with vector only or t*Q(UUG)L,* t*K(UUU)L* overexpression construct. Transformants were tested for growth on YPD 40^{o}C and YPD+2mM caffeine 40^{o}C using a dilution series for three days and compared to growth of an isogenic wildtype strain. B) Vector only, wildtype *ELP1* single copy construct and t*Q(UUG)L,* t*K(UUU)L* overexpression constructs were transformed into a *sec2-59 elpΔKAN* strain. Transformants were tested for growth on SC-leucine media at 25^{o}C and 32^{o}C for three days.


A)
YPD 40°C
YPD+2mM Caff 40°C
Wildtype
elp1Δ
elp2Δ
elp3Δ
Wildtype
elp4Δ
elp5Δ
elp6Δ
+ pRS425
+ tQ (UUG) tK(UUU) pRS425
B)
SC-Leu 25°C
SC-Leu 32°C
sec2-59, elp1Δ
+ pRS425
+ ELP1
+ tQ(UUG)L, tK(UUU)L pRS425

work out this discrepancy, but it may be due to construct variability, strain differences, tRNA expression levels or another technical reason.

Additional phenotypic analysis to focus the list of processes that phenocopy Elongator

Approximately 45 genes were identified from our screen that are required for growth on YPD + 2mM caffeine at 40°C, but not 2mM caffeine at 37°C or YPD at 40°C. These proteins function in a variety of cellular processes. Therefore, it was still difficult to narrow the cellular process that phenocopies loss of Elongator function. We reasoned an additional phenotype that could be tested is suppression of caffeine sensitivity at 40°C through overexpression of t*Q(UUG)L* and t*K(UUU)L*.

Suppression of caffeine sensitivity was tested for all 45 genes identified in the caffeine sensitivity screen. This phenotype is suppressed through overexpression of t*Q(UUG)L* and t*K(UUU)L* in six strains: *elp2Δ, elp4Δ, elp6Δ, kti12Δ, ats1/kti13Δ* and *swf1Δ*. An example of this analysis is shown in Figure 5.10. Kti12p and Ats1/Kti13p are two proteins that have been reported to phenocopy and associate with Elongator, therefore this result was not surprising [3, 17, 51-53]. Swf1p is a SNARE palymitoyltransferase. Therefore, two functions were isolated that display this phenotype: Elongator function and SNARE modification.

Kti11p, Kti12p and Kti13p function with Elongator in exocytosis regulation

Caffeine sensitive genes were tested for genetic interactions with secretory mutants *sec2-59* and *sec15-1*. Deletion of *kti12* and *ats1/kti13* were tested, as well as *kti11*, for genetic interactions with *sec2-59* and *sec15-1*. *KTI11* encodes for a small, highly conserved zinc-finger protein that has been shown to function in multiple processes and associates with Elongator [3, 16, 52, 54-56]. Deletion of *kti11, kti12*

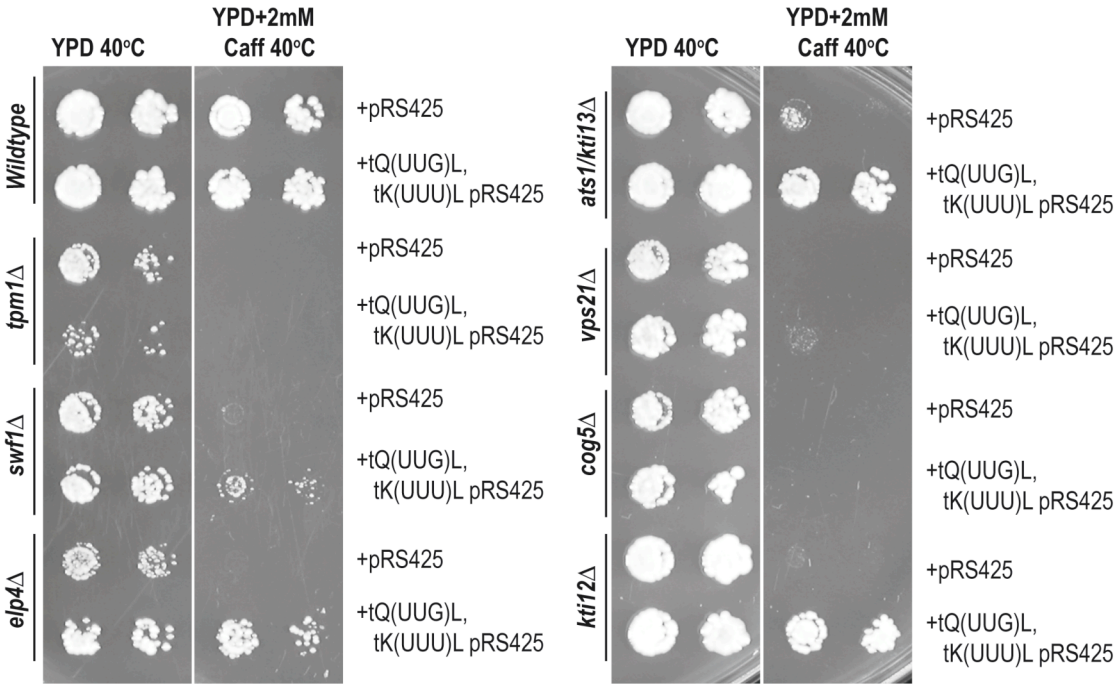


Figure 5.10. Suppression of caffeine sensitivity through overexpression of two tRNA species. An example analysis of cells with a deletion of a caffeine sensitive strain was transformed with vector only or t*Q(UUG)L,* t*K(UUU)L* overexpression construct. Transformants were tested for growth on YPD 40°C and YPD+2mM caffeine 40°C using a dilution series for three days and compared to growth of an isogenic wildtype strain.

and *ats1/kti13* suppress *sec2-59* and *sec15-1* to a similar extent as loss of Elongator function (Figure 5.11). This suggests that Elongator negatively regulates exocytosis and Kti11p, Kti12p and Ats1/Kti13p may mediate the regulation. These three proteins have been identified to perform multiple functions *in vivo*. Kti11p and Kti12p have been shown {Kholodenko, 2003 #149}to associate with Elongator and influence its function [15, 52, 55]. Ats1/Kti13p was originally identified as a high copy suppressor of an α-tubulin mutant and was suggested to function in microtubule maintenance and regulate bud morphology through interacting with Nap1p [57, 58]. Kti11p, Kti12p and Ats1/Kti13p are required with Elongator to generate modified uridine species

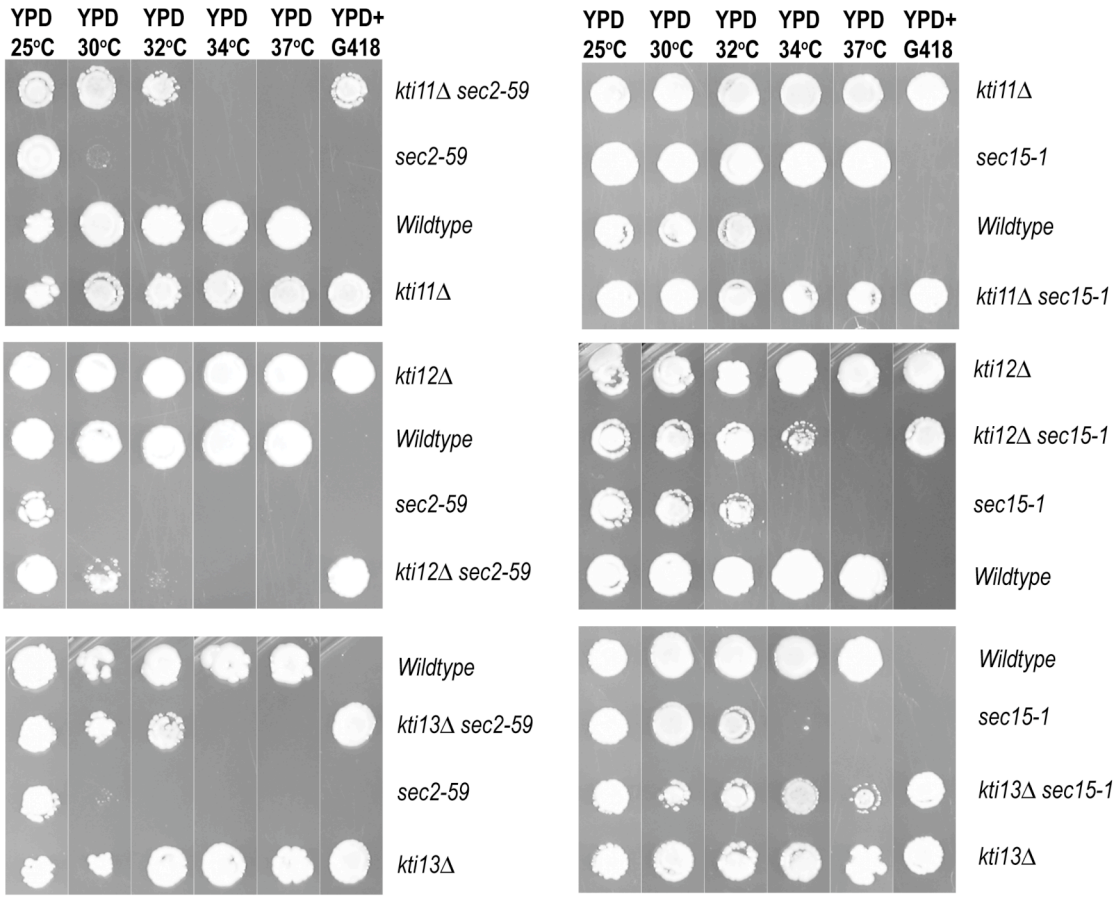


Figure 5.11. Kti11p, Kti12p, and Kti13p negatively regulate exocytosis. Strains containing *kti11Δ*, *kti12Δ*, or *kti13Δ* were crossed with a mutant strain containing *sec2-59* (left column) or *sec15-1* (right column). Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

[3, 51]. Kti11p is a small protein that has been implicated in a variety of functions including secretion, Elongator function and dipthamide biosynthesis [16, 52, 56].

Production of the translation elongator factor, Eft2p, requires dipthamide. We wished to determine if loss of Elongator and Kti11p function resulted in loss of Eft2p, and due to a decrease in cellular dipthamide levels Kti11p, negatively regulates exocytosis at the translational level. Deletion of *EFT2* does not suppress *sec2-59* or *sec15-1,* indicating that Elongator and Elongator-associated proteins regulate

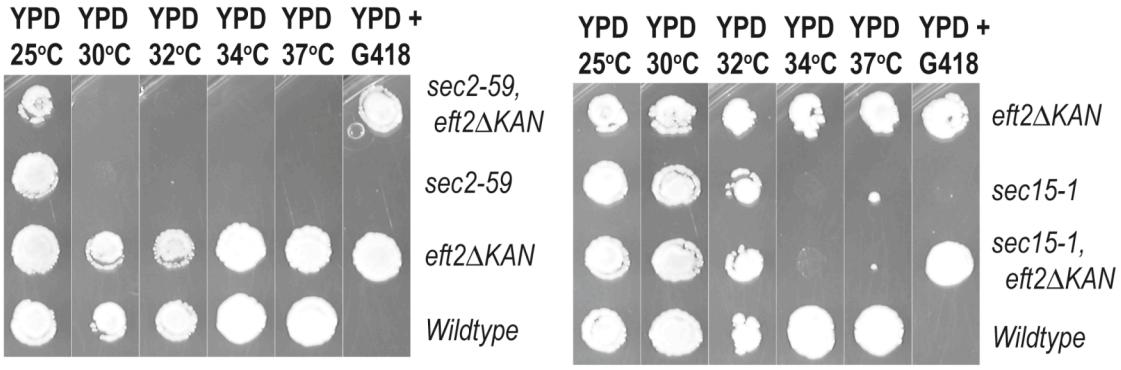


Figure 5.12. Elongator negatively regulates exocytosis independently of diphthamide-containing protein *EFT2*. A strain containing *eft2ΔKAN*^{R} was crossed with *sec2-59* and *sec15-1*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

exocytosis independently of diphthamide biosynthesis and Eft2p (Figure 5.12). Supporting this, Kti11p functions in diphthamide biosynthesis independently of Elongator function, as cells lacking Elongator have normal cellular levels of diphthamide [59]. Trm9p is required for tRNA modifications downstream of Elongator and to determine if this is a mode of regulation at the translational stage, *trm9Δ* was crossed with *sec15-1* [60]. Figure 5.13 shows that deletion of *trm9* does not suppress *sec15-1* mutants. The data presented above suggest that Elongator, Kti11p, Kti12p and Kti13p negatively regulate exocytosis independently of diphthamide biosynthesis and Eft2p and independently of *TRM9*-dependent tRNA modifications.

Genetic Analysis of caffeine sensitive genes

Genetic analysis using a subset of the caffeine sensitive genes was used to try to identify cellular processes that negatively regulate exocytosis. These genes were crossed with mutants of the Rab exchange factor *sec2-59* and with a Rab effector *sec15-1* to analyze for genetic interactions at both the Rab activation step and Rab

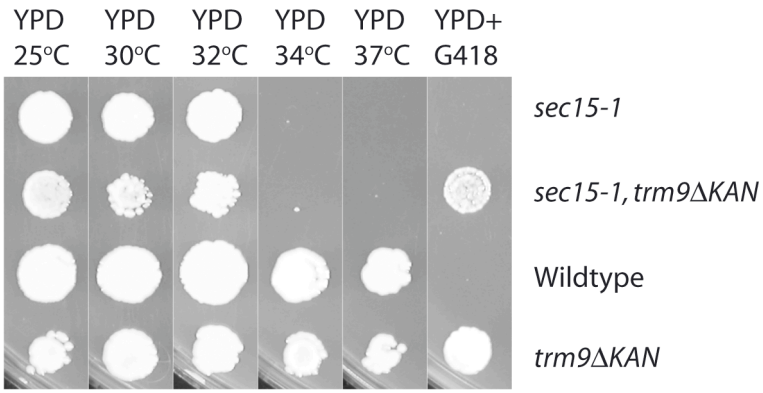


Figure 5.13. Elongator negatively regulates exocytosis independently of *TRM9*-dependent tRNA modifications. A strain containing *trm9ΔKAN*^{R} was crossed with *sec15-1*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

targeting at the plasma membrane. Table 5.5 displays a summary of the genetic interactions between the genes listed and *sec2-59* and *sec15-1*.

TOR1 does not appear to directly influence exocytosis as deletion does not suppress the temperature sensitivity of *sec2-59*. In addition, growth of *sec2-59* mutants are unaffected by the presence of rapamycin (data not shown). TOR signaling does influence cell growth and proliferation but this data suggests that Tor1p does not directly influence exocytosis through Sec2p to provide membrane to the cell surface for membrane expansion.

Chs7p- and Vps64p-dependent protein sorting is not required for negative regulation of exocytosis, as deletion of these genes does not affect *sec2-59* or *sec15-1* temperature sensitivity (Figure 5.14). Deletion of *vps21* was tested to identify a connection between Vps21p-dependent endocytosis and exocytosis. Deletion of *vma8* was tested to determine if loss of organelle acidification suppresses exocytic mutants. Loss of Vps21p-dependent transport and defects in organelle pH regulation through deletion of *vma8* does not suppress exocytic mutants.

Table 5.5. Summary of genetic interactions between caffeine sensitive genes and *sec2-59* or *sec15-1* mutants. (++) strong suppression, (+) suppression, (+/-) weak suppression, (syn lethal) synthetic lethal interaction, (-) no genetic interaction.

	Cellular Role	Genetic Interactions	
		sec2-59	*sec15-1*
*elp1*Δ	Elongator subunit	++	++
*kti11*Δ	Elongator accessory protein	++	++
*kti12*Δ	Elongator accessory protein	++	++
*kti13/ats1*Δ	Elongator accessory protein regulates microtubules and cell cylce	++	++
*vps64*Δ	required for cytoplasm to vacuole protein transport	–	–
*vps21*Δ	endocytic Rab GTPase	–	–
*gtr1*Δ	small GTPase required for signal-mediated traffic of amino acid transporters	–	++
*cog5*Δ	subunit of Golgi tethering complex - COG Complex	++	–
*vma8*Δ	Role in vacuolar acidification	–	–
*chs7*Δ	regulates Chs3p export from ER	–	–
*swf1*Δ	SNARE palmitoyltransferase	syn lethal	syn lethal
*tor1*Δ	Target of Rapamycin - kinase that regulates cell growth in response to nutrients	–	not tested

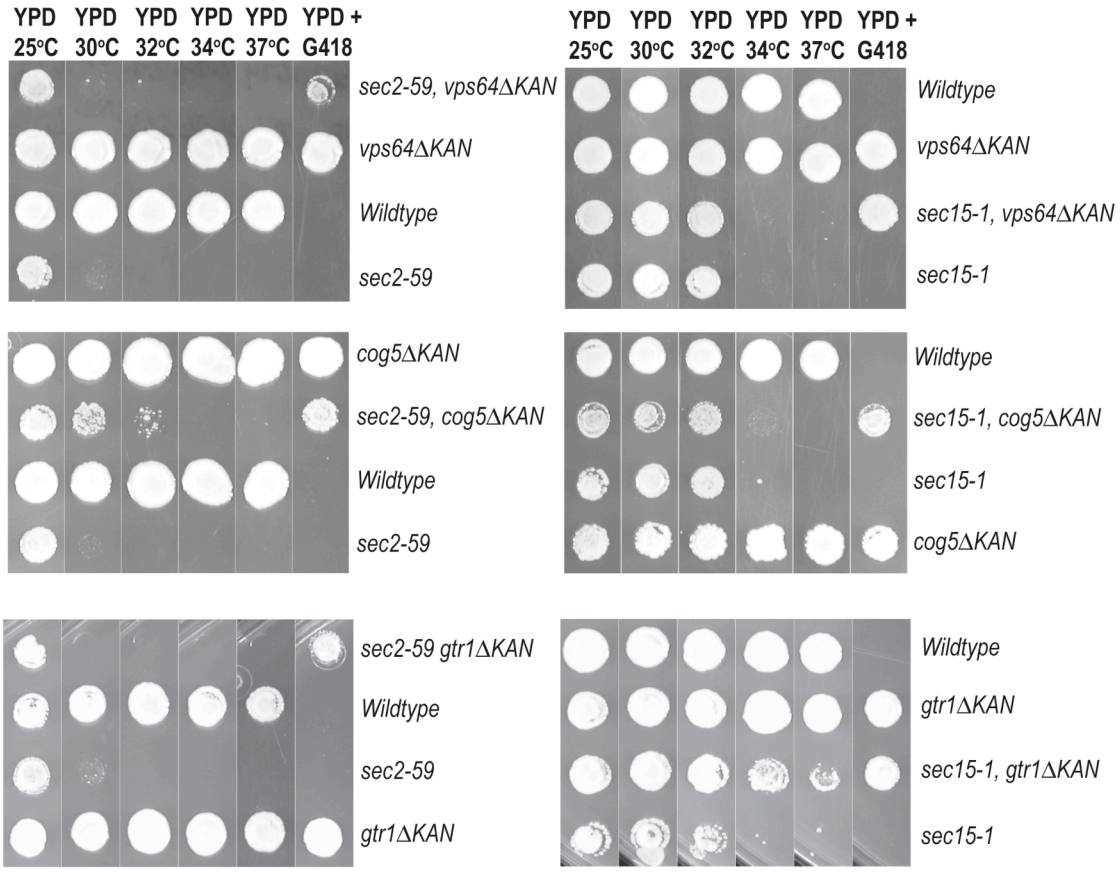


Figure 5.14. *COG5* and *GTR1* function influences exocytic events. Strains containing *cog5Δ*, *gtr1Δ vps64/far9Δ* were crossed with a mutant strain containing *sec2-59* (left column) or *sec15-1* (right column). Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

Disruption of the COG complex, more specifically lobe B of the complex, through deleting *COG5*, suppresses *sec2-59* mutants but not *sec15-1* mutants (Figure 5.14) [45]. Deletion of *GTR1,* which disrupts the sorting of Gap1p and potentially other endosomal transport steps, suppresses *sec15-1* mutants but not *sec2-59* mutants. These data suggest that tethering events at the Golgi and protein sorting in the endosomes may influence specific exocytic events.

Swf1p has a direct role in regulating membrane transport and shares Elongator phenotypes

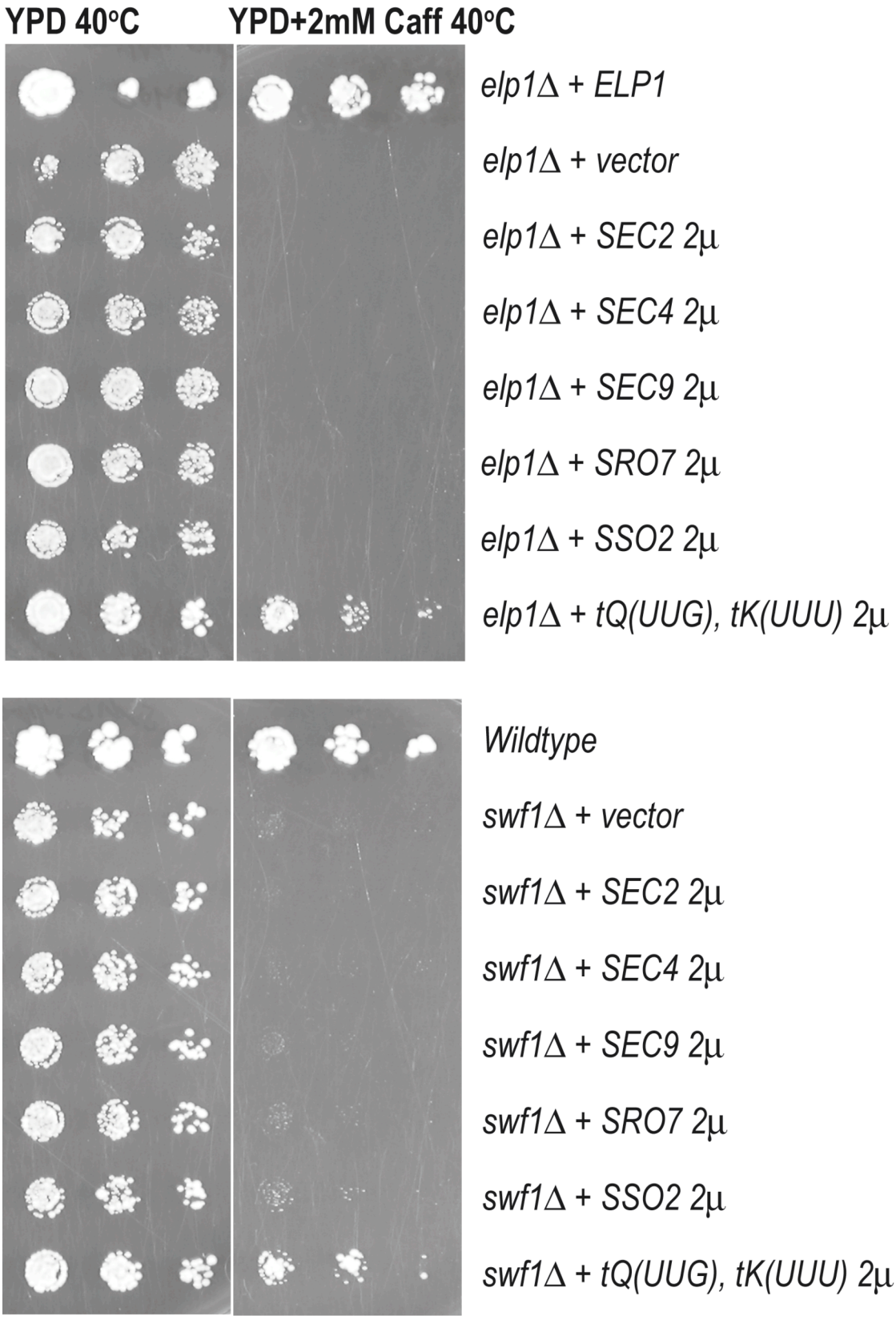
SWF1 was identified in the caffeine screen as a gene required for growth on 2mM caffeine at 40^{o}C. In addition to sharing this phenotype with Elongator, it also phenocopies the more specific phenotype where caffeine sensitivity can be partially suppressed through overexpression of t*Q(UUG)L*, t*K(UUU)L* tRNA species (Figure 5.10). Swf1p is a SNARE palmitoyltransferase and has a direct role in membrane transport through regulating SNARE stability [32, 33]. Deletion of *SWF1* with either *sec-59* or *sec15-1* mutations results in an inviable cell (Table 5.5).

Therefore, *SWF1* shares three phenotypes with Elongator genes as they genetically interacts with *sec* mutants, they are caffeine sensitive at 40^{o}C and the caffeine sensitivity can be suppressed through overexpression of t*Q(UUG)L* and t*K(UUU)L*. Genetic analysis discussed earlier indicates that loss of Elongator function results in a similar suppression profile to overexpression of *SEC4,* the t-SNARE *SEC9* and the effector *SRO7*. We wondered if overexpression of positive regulators of exocytosis could also suppress the caffeine sensitivity of *elp1Δ* or *swf1Δ* perhaps providing a link between exocytosis and the tRNA species. Overexpression of *SEC2, SEC4, SEC9, SRO7* or the t-SNARE *SSO2* could not suppress the caffeine sensitivity of *swf1Δ* or *elp1Δ* (Figure 5.15). Therefore, the suppression must occur through a mechanism other than providing excess protein of the regulators tested.

Discussion

A genome-wide phenotypic analysis identified many proteins involved in membrane transport that, like Elongator, are required for growth on YPD + 2mM caffeine at 40^{o}C, but not required for growth on YPD + 2mM caffeine at 37^{o}C or YPD at 40^{o}C. Multiple proteins involved in protein sorting, vesicle transport and targeting

Figure 5.15. Suppression analysis of *swf1*Δ and *elp1*Δ induced caffeine sensitivity. A strain containing *elp1*Δ (top) or *swf1*Δ (bottom) was transformed with the indicated overexpression plasmid. Transformants were tested for growth on YPD 40°C and YPD+2mM caffeine 40°C using a dilution series for three days.


YPD 40°C
YPD+2mM Caff 40°C
elp1Δ + ELP1
elp1Δ + vector
elp1Δ + SEC2 2μ
elp1Δ + SEC4 2μ
elp1Δ + SEC9 2μ
elp1Δ + SRO7 2μ
elp1Δ + SSO2 2μ
elp1Δ + tQ(UUG), tK(UUU) 2μ
Wildtype
swf1Δ + vector
swf1Δ + SEC2 2μ
swf1Δ + SEC4 2μ
swf1Δ + SEC9 2μ
swf1Δ + SRO7 2μ
swf1Δ + SSO2 2μ
swf1Δ + tQ(UUG), tK(UUU) 2μ

and organelle pH regulation are required, suggesting a physiological link between these processes and Elongator function.

Two independent analyses suggest an *in vivo* connection between Elongator function and SNARE activity. Suppression analysis of exocytic mutants shows that loss of Elongator function is similar to overexpression of the exocytic t-SNARE *SEC9,* the Rab GTPase *SEC4* and its effector *SRO7*. Also, a genome-wide screen identified *SWF1* as one of six genes that 1) is required for growth on 2mM caffeine at 40^{o}C and 2) is suppressed by overexpression of two tRNA species.

Overexpression of t*Q(UUG)L* and t*K(UUU)L* bypasses the caffeine sensitivity of Elongator and *SWF1* deletion, demonstrating a physiologic connection between regulation of membrane transport and translation. These data suggest that the tRNA species may act downstream of Elongator and Swf1p function, although this has yet to be determined. The nature of how overexpression of the tRNA species suppresses this phenotype is unclear.

Swf1p palmitoylates multiple SNAREs *in vivo* and loss of this modification causes ubiquitination and degradation of the SNARE [32, 33]. One hypothesis is that Elongator may negatively regulate exocytosis through regulating the availability of Sec9p at the plasma membrane as suggested by the genetic analysis provided in this study. Sec9p does not contain a transmembrane domain or cysteine residues required for palmitoylation [61]. It associates with the plasma membrane through interacting with the t-SNARE Sso1p or Sso2p. Therefore we sought to investigate the amount of Sso2p in membranes in wildtype, *elp1Δ,* and *swf1Δ* cells as this might suggest the status of Sec9p at the membrane. We reasoned that the genetic data could be explained, perhaps, through excess SNARE available at the plasma membrane in *elp1*Δ cells. In this scenario loss of Elongator function would result in excess

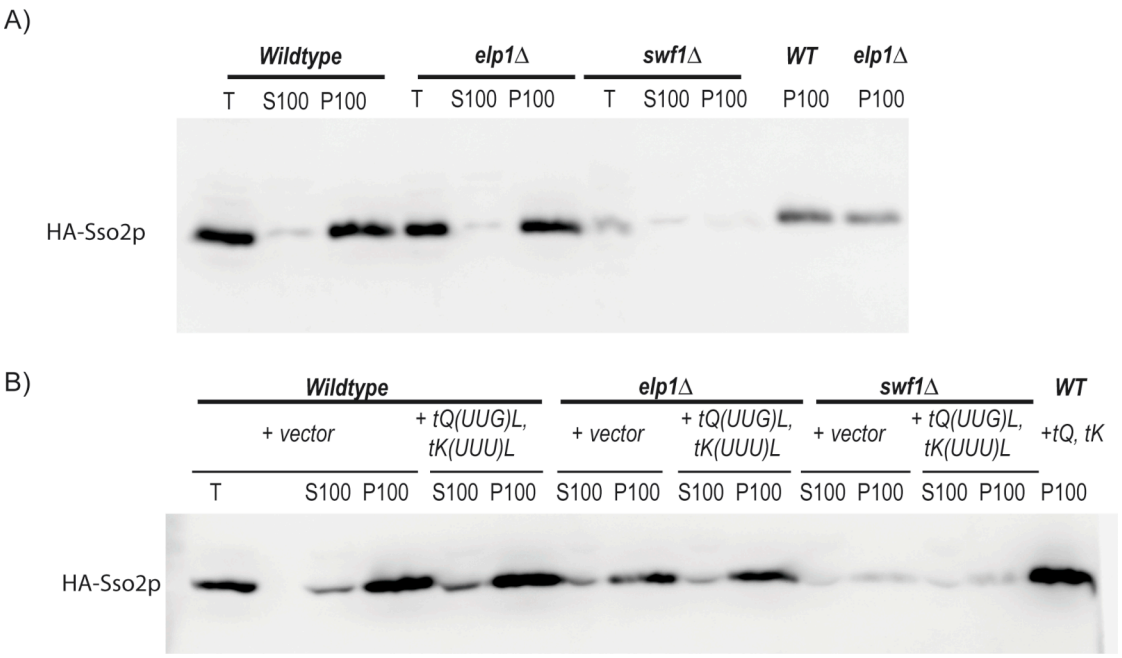


Figure 5.16. *swf1Δ* cells are defective in Sso2p levels on membranes but overexpression of t*Q(UUG)L*, t*K(UUU)L* does not influence this defect. A) HA-Sso2p cellular fractionation was tested in isogenic wildtype, *elp1Δ* and *swf1Δ* cells. B) HA-Sso2p cellular fractionation was testing in the above strains also containing vector only or t*Q(UUG)L,* t*K(UUU)L* overexpressed. T is total HA-Sso2p in cells, S100 is HA-Sso2p in supernatant fraction after 100,000xg centrifugation, and P100 is HA-Sso2p in pellet fraction after 100,000xg centrifugation. HA-Sso2p was detected using a polyclonal αHA antibody.

available SNAREs, particularly Sec9p, and therefore have a similar suppression profile of post-Golgi mutants as overexpression of *SEC9*.

Both wildtype and *elp1Δ* cells have comparable levels of Sso2p in the membrane fraction (P100) (Figure 5.16a). Loss of *SWF1* results in drastically reduced levels of Sso2p in the membrane fraction, presumably because of ubiquitination and degradation of the protein [32]. This defect, however, could not be rescued with overexpression of t*Q(UUG)L* and t*K(UUU)L* (Figure 5.16b), indicating that 1) the caffeine sensitivity is not due to a defect in Sso2p levels at the membrane and 2) that the suppression of caffeine sensitivity with tRNA species is unrelated to levels of Sso2p at the membrane. Also, overexpression of the tRNA species does not simply

overexpress *SEC9* or other positive regulators of this pathway, as overexpression of these proteins does not suppress the caffeine sensitivity of these proteins. Therefore, overexpression of t*Q(UUG)L* and t*K(UUU)L* suppresses the caffeine sensitivity of *swf1Δ* through another mechanism.

Genetic analysis demonstrates that deletion of *SWF1* in concert with *sec2-59* and *sec15-1* is lethal to the cell. This result is not surprising as Swf1p regulates SNARE function and stability [32, 33]. The exocytic SNAREs, Snc1p and Snc2p (v-SNARE), and Sso1 and Sso2 (t-SNARE), that promote exocytosis are substrates for Swf1p [32, 33, 62]. Therefore globally reducing SNARE function would have negative effects on exocytosis. Taking this data with other phenotypic data, *SWF1* and Elongator share the phenotype that loss of function results in caffeine sensitivity and the caffeine sensitivity can be suppressed through overepxression of tRNA species. Genetically *SWF1* and Elongator have opposite phenotypes where deletion of *SWF1* is synthetic lethal with late *sec* mutants and deletion of *ELP* suppresses late *sec* mutants.

The connection between translation and exocytosis still remains unclear. Elongator-dependent modification of uridine promotes translation; therefore loss of Elongator function should not promote overexpresssion of proteins. Furthermore, loss of Elongator function suppresses multiple mutants in the pathway and the physiological effect of losing Elongator function does not simply read through a stop codon. Previously reported studies have demonstrated a link between exocytosis and translation. The exocyst physically interacts with the Sec61p subunit of the translocon [63-65]. *In vivo* analysis verifies a physiologically relevant interaction. The translocon interacts directly with ribosome subunits through the Sec61p subunit to ensure that newly synthesized transmembrane proteins are passed across the ER lipid bilayer. Furthermore, overexpression of the exocyst subunit Sec10 in mammalian cells leads to

an increase in protein synthesis of secreted proteins despite constant mRNA levels, indicating secretion can influence protein synthesis [64, 66].

Alternatively, perhaps modification of tRNA species generates a molecule that influences membrane transport and exocytosis. Overexpression of the tRNA species may have a kinetic relationship with regulators of membrane transport perhaps through competing for binding sites or substrate. The SAM radical domain on Elp3p could potentially perform a variety of reactions and may be responsible for generating modifications on tRNA molecules [26]. A recent report also suggests a role for SAM-dependent methylation in chemotaxing *Dictyostelium*, which can be thought of as analogous to the budding process in yeast. In accordance with this, Elp3p requires a functional SAM radical domain to negatively regulate exocytosis (I. Berke and R. Collins, unpublished data).

Phenotypic and biochemical data show that holo-Elongator consists of six subunits and Kti11-13p also associates to facilitate Elongator function [10, 11, 15-17, 55]. Genetic interaction data indicates that holo-Elongator function regulates exocytosis and Kti11p, Kti12p and Kti13p facilitate this regulation. Elp1p and Elp2p have predicted WD-40 motifs and may function as scaffold proteins [67]. Elp3p contains two potential catalytic activities, acetyltransferase and SAM radical activity, that are the only known catalytic functions of Elongator to date [26, 68]. It is possible that Elongator subunits and Elongator-associated proteins recruit different substrates for Elp3p and through this influences multiple pathways. In human cells, Kti11 and Kti13 have also been shown to influence secretion [69-71]. A human homolog of Kti13p (DelGEF) was shown to interact with Exocyst subunit Sec5 and the homolog of Kti11p (DelGIP) enhances this interaction [69-71]. Similar to our studies in yeast, these reports also revealed that knocking down DelGIP or DelGEF levels using RNA interference resulted in an increase in proteoglycan secretion. Thus, in humans Kti11

and Kti13 homologous proteins also appear to negatively regulate certain aspects of exocytosis.

Our data strengthens the role of Elongator's function in regulating membrane transport and provides a broad picture of how it affects exocytic mutants. The direct connection between translation and exocytosis remain unclear but the *SWF1* and *ELP* phenocopy on caffeine demonstrates a physiological link between regulation of SNARE function and Elongator function. Our current model suggests Elongator may influence the ability of Sec4p to act on the t-SNARE Sec9p.

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Chapter 6. Deacetylase Localization in *S.cerevisae*

Abstract

While it is well established that protein acetylation can modulate function in the nucleus, its role in cytoplasmic processes is just beginning to be understood. Acetylation requires acetyltransferase activity to attach an acetyl group to its substrate and deacetylases to remove the acetyl group. Similar to phosphorylation, acetylation provides a reversible modification that alters protein function. Previous work in our laboratory identified a role for acetylation in the regulation of exocytosis in an Elp3p-dependent manner. This suggests that one or more regulators of this process may be acetylated. This study reports the localization of the ten deacetylases in yeast and finds that cells contain multiple cytoplasmic deacetylases, including Hos3p, which forms a collar at the interface between the mother and daughter cells.

Introduction

Protein function can be regulated transcriptionally and translationally through controlling intracellular protein levels, but also posttranslationally with a variety of modifications that influence protein function. Posttranslational modifications can influence protein function to a specific cellular space, for example, through addition of acyl groups to create a hydrophobic environment to attract proteins to membranes. This occurs in the human oncogene Ras, where acylation targets Ras to the plasma membrane to transmit signals at the membrane [1]. Other modifications, such as phosphorylation, can structurally and functionally alter proteins, which could reveal an active site or change the environment or architecture of a binding face.

Acetylation is a protein modification with well-established roles in the nucleus that drastically affects gene expression patterns. Non-nuclear roles for acetylation are

just becoming appreciated. This version of acetylation is different from the co-translational process of N-terminal acetylation by NatA, NatB and NatC acetyltransferases that can influence protein function [2]. Recently, it was shown that acetylation of MEK2 (Map kinase kinase) and IκB kinase (IKK) regulates protein activity [3]. This study found that the *Yersinia* virulence factor, YopJ, which inhibits the host inflammatory response, acetylates MEK2 and IKK and prevents the phosphorylation of these proteins, which is required for signal propagation. The authors postulated these proteins may also be regulated through acetylation under nonpathogenic conditions and that cytoplasmic acetylation may have a broad impact on protein function. A recent proteomic study identified many cytoplasmic targets of lysine acetylation, further implicating a role for acetylation in regulating non-nuclear processes [4]. For example, two proteins that impact Rho GTPase function were identified as substrates for lysine acetylation: RhoGDI and p120 catenin. This study found that RhoGDI is acetylated at residues that may play an inhibitory role or alter its G-protein specificity.

Similar to phosphorylation, which uses a reversible cycle governed by kinase (phosphorylate) and phosphatase (dephosphorylate) enzymes, acetylation undergoes cyclical regulation. Acetyltransferases catalyze the transfer of an acetyl group from acetyl CoA to an amino acid or small molecule. Deacetylases then remove the acetyl group from this site and the protein or molecule returns to its deacetylated state. The *S. cerevisae* genome encodes for at least 10 deacetylases that can be grouped into three classes based on primary sequence homology (Figure 6.1) [5].

Studies in our laboratory found a role for Elp3p-dependent acetylation in the regulation of exocytosis [6]. The nature of how this reversible modification affects exocytosis is unclear. One possible scenario is that Elp3p acetylates a regulator of exocytosis and this modification negatively regulates its function. Acetyl-lysine

Class I	Class II	Class III
Rpd3	Hos3	Hst2
Hos2	Hda1	Hst3
Hos1		Hst4
		Sir2
		Hst1

Figure 6.1. Deacetylases in *S.cerevisiae*. The deacetylase enzymes in yeast classified into three classes based on primary sequence analysis. Adapted from [5].

antibodies are excellent for analyzing the state of modified histones but are not as sensitive for detecting other acetylated proteins, such as cytoplasmic proteins. Because acetylation is a highly reversible modification, identifying the deacetylase responsible for the regulation might aid in the development of an assay for detection of relevant cytoplasmic targets. This analysis characterizes the localization of GFP-tagged deacetylases.

Materials and Methods

Yeast strains, plasmids and media

Yeast strains used in this study are listed in Table 1 and plasmids used are in shown Table 2. YPD (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), and SD (0.1% yeast nitrogen base, 2% D-glucose, plus required nutrients) media was used.

Table 6.1. Yeast strains used in this study:

Strain number	Genotype
RCY239	Mat **a** *ura3*
RCY2432	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 XPO1Δ::LEU2 [xpo1-1 HIS3 CEN]*
RCY2433	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0*
RCY4053*	*MAT***a** *ura3 leu2 his3*
M-239	*MAT***a** *ura3 leu2 his3 trp1 cdc12-6*

* isogenic control for *cdc12-6* generated through isolating temperature resistant spores from crossing Longtine laboratory strains M-12(*MATα ura3 leu2 cdc3-3*) x M195(*MAT***a** *ura3 leu2 his3 cdc10-1*).

Construction of GFP-tagged proteins

GFP-tagged deacetylase proteins were generated on episomal shuttle plasmids (pRS315) using overlap PCR and homologous recombination. Each protein was tagged at its C-terminus with GFP followed by the *ADH1* terminator. A linker region, consisting of amino acids SAGGSASAGGPGG where S is serine, A is alanine, G is glycine and P is proline, is located between the 3' end of the coding sequence (just prior to endogenous stop codon) and GFP. Protein expression is controlled by the endogenous promoter where approximately 500 to 600 basepairs upstream of the coding sequence is present in the construct. Restriction enzyme digestion was used to verify the construct.

Microscopy

Steady state localization and localization in *xpo1-1* mutants, images were taken using the 60X lens because a number of the deacetylase proteins are expessed at such low levels. Differential interference contrast (DIC) images, FITC images and TxRed images were taken at one plane. RFP-tagged proteins were analyzed with a TxRed filter (excited at 540-580nm and emission at 600-660nm). GFP-tagged proteins were analyzed with a FITC filter (excitation at 465-495nm and emission at 515-555). Live

Table 6.2. Plasmids used in this study:

Plasmid number	Plasmid	Source
pRC1166	*NLS-NES-GFP-GFP* pRS426	Silver laboratory
pRC2601	*RPB10-RFP* pRS315	[6]
pRC3664	*HOS3-GFP* pRS316	This study
pRC3665	*RPD3-GFP* pRS316	This study
pRC3745	*HST2-GFP* pRS316	This study
pRC3746	*HST3-GFP* pRS316	This study
pRC3799	*HOS2-GFP* pRS316	This study
pRC3800	*HDA1-GFP* pRS316	This study
pRC3801a	*HST1-GFP* pRS316	This study
pRC3802a	*HST4-GFP* pRS316	This study
pRC3803a	*SIR2-GFP* pRS316	This study
pRC3804	*HOS1-GFP* pRS316	This study

cells were analyzed with a Nikon Eclipse E600 microscope 60X (1.4NA) lens, 1X optavar (0.110 μm/pixel) and imaged using a Sensicam EM High Performance camera (The Cook Corporation).

To analyze localization in exportin mutant cells, *xpo1-1* and wildtype cells, containing each GFP-tagged cytosolic deacetylase, were grown overnight at room temperature in minimal media. Cells were temperature shifted to 37^{o}C for one hour. Localization was analyzed at room temperature and 37^{o}C.

For analysis of Hos3p-GFP localization, fluorescent images were collected as a z-series with a 0.2μm z step size using a 100X (1.4NA) lens, 1X optavar (0.080 μm/pixel). DIC images were taken in one plane. Images were captured with IP Lab 3.6.5 software and blind deconvolution with 30 iterations was done using Autodeblur and Autovisualize 9.1 software. 3-dimensional reconstruction of the z-series and further analysis was performed using Imaris program.

Hos3p-GFP localization was analyzed in septin mutant cells. Wildtype and *cdc12-6* cells containing Hos3p-GFP were grown overnight in synthetic complete

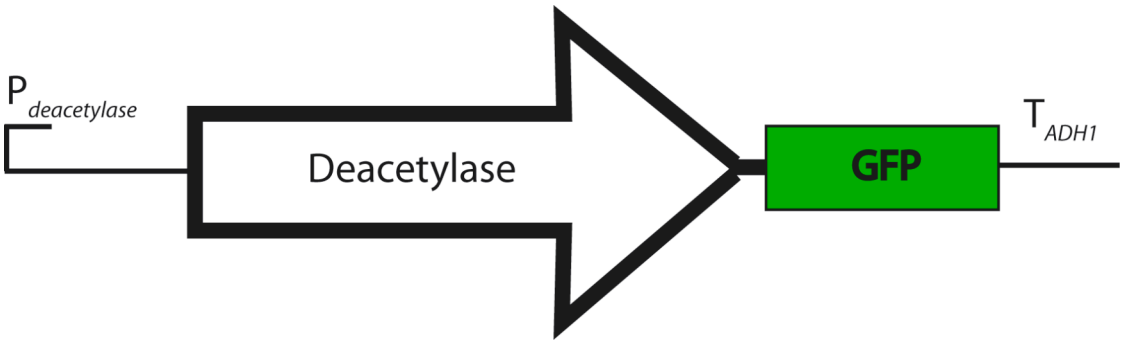


Figure 6.2. Strategy for generating deacetylase-GFP fusion proteins. Each deacetylase was tagged at the C-terminus into single copy shuttle plasmids. Protein expression was controlled by the endogenous promoter and termination was controlled by the *ADH1* terminator.

minus uracil at room temperature. Localization was analyzed after growth at 37^{o}C for 0, 1, 2 and 3 hours.

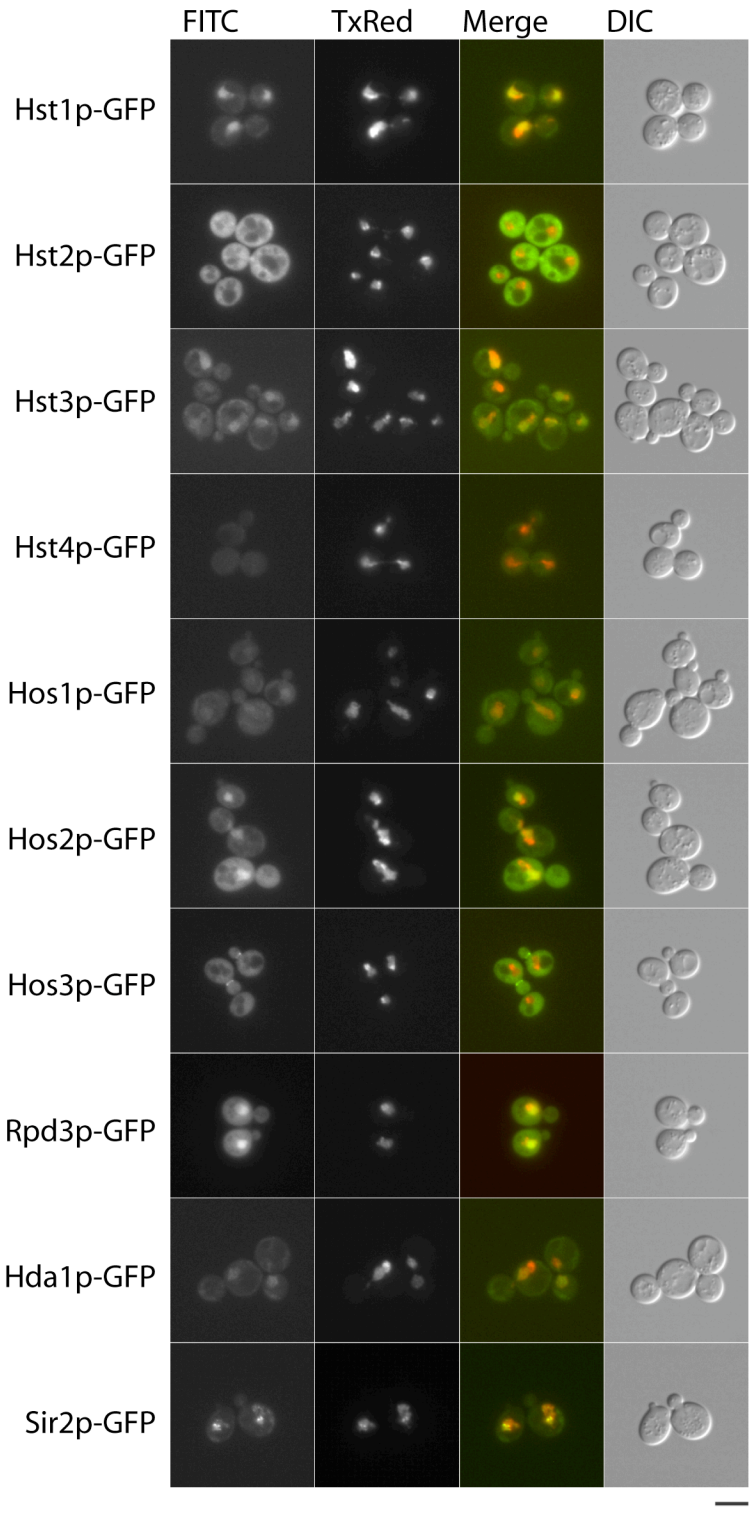
Results

Steady-state localization of the deacetylase enzymes in S.cerevisae

Work discussed in Chapters 4 and 5 established a role for Elongator Complex function in the regulation of exocytosis [6]. One of the proposed catalytic activities of Elongator is acetyltransferase activity, which it has been shown to possess *in vitro* on histone tails [7, 8]. The *in vivo* target of Elp3p acetylation may not be histones as it is localized to the cytoplasm and does not shuttle between the nucleus and cytoplasm [6, 9]. In an attempt to try to identify the relevant targets of Elp3p acetylation, we sought to initially determine the localization of the deacetylase enzymes in yeast to determine the cytoplasmically localized enzymes, which may be the physiologically relevant counterpart of Elp3p-dependent acetylation.

Each deacetylase was tagged with GFP at the C-terminus into a single copy shuttle plasmid (Figure 6.2). The expression of each fusion protein was under the

Figure 6.3. Steady-state localization of GFP-tagged deacetylases in wildtype cells. Steady-state localization in wildtype cells after growth in minimal media at room temperature. Rpb10p-RFP is used as a nuclear marker for colocalization analysis. The corresponding differential interference contrast (DIC) images are shown. Size bar is 5μm.


FITC
TxRed
Merge
DIC
Hst1p-GFP
Hst2p-GFP
Hst3p-GFP
Hst4p-GFP
Hos1p-GFP
Hos2p-GFP
Hos3p-GFP
Rpd3p-GFP
Hda1p-GFP
Sir2p-GFP

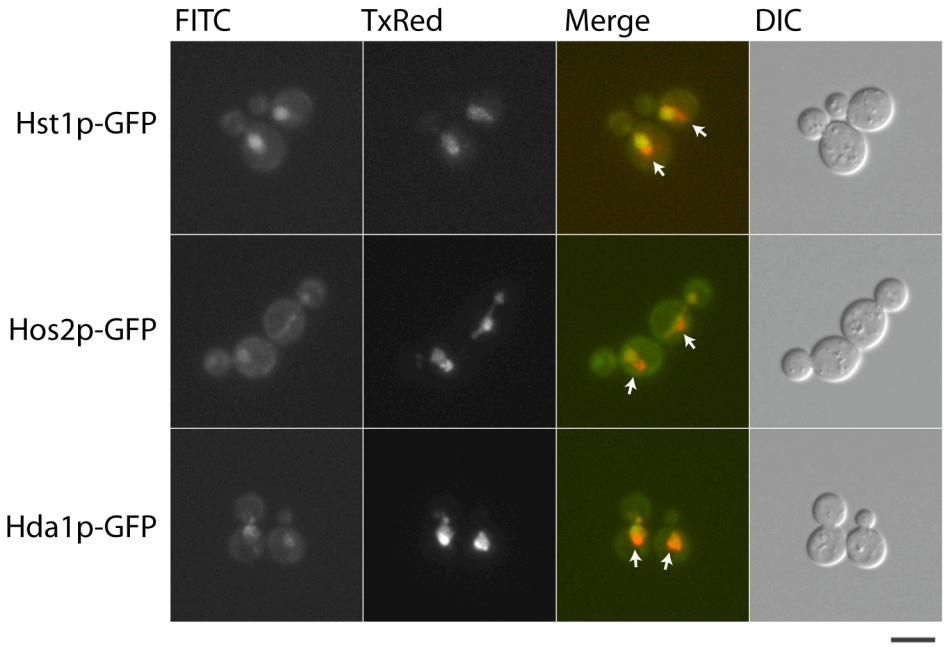


Figure 6.4. Nuclear partitioning of a subset of nuclear deacetylases. Hst1p-GFP, Hos2p-GFP and Hda1p-GFP colocalization with the nuclear marker Rpb10p-RFP. Arrows indicate regions that appears to contain only Rpb10p-RFP, but not the GFP tagged deacetylase. The corresponding DIC images are shown. Size bar is 5μm.

control of its endogenous promoter to ensure that localization was not altered due to overexpression. Each tagged protein was tested for localization in wildtype cells. RFP-Rpb10p, an RNA polymerase II subunit, was used to determine the localization of the nucleus in each cell [10].

Overall, expression levels of many of the deacetylases are relatively low. This can be assessed simply through the relative GFP signal intensity in the live cells expressing each individual protein. We found through colocalization studies that Hst1p-, Hst3p-, Hos1p-, Hos2p-, Rpd3p-, Had1p- and Sir2p-GFP localize to the nucleus (Figure 6.3). Interestingly, using RFP-Rpb10p as a nuclear marker, some deacetylases appear to be concentrated to a specific region of the nucleus (Figure 6.4).

S.cerevisiae Deacetylase Localization

Deacetylase	Localization		
	Nucleus	Cytoplasm	Neck
Hst1p	X		
Hst2p		X	
Hst3p	X		
Hst4p		X	
Hos1p	X	X	
Hos2p	X	X	
Hos3p		X	X
Rpd3p	X	X	
Hda1p	X		
Sir2p	X		

Figure 6.5. Summary of GFP-deacetylase steady-state localization. Steady-state localization as assessed in Figure 6.3.

Rpb10p is a subunit of RNA polymerase II and therefore would be present at regions in the nucleus of active transcription. The compartmentalization of certain deacetylases may be reflective of the subnuclear region where each protein functions.

Hst2p-, Hst4p-, Hos1p-, Hos2p-, Hos3p- and Rpd3p-GFP proteins showed a cytoplasmic distribution at varying levels of expression (Figure 6.3). Therefore, Hos1p-, Hos2p- and Rpd3p-GFP localize to both the nucleus and cytoplasm. Hst2p-GFP appears to be abundantly expressed, solely in the cytoplasm. Hos3p-GFP is unique among the deacetylases as, in addition to a cytoplasmic distribution, it also concentrates at the neck between the mother and daughter cell. A summary of the localizations of each deacetylase is shown in Figure 6.5.

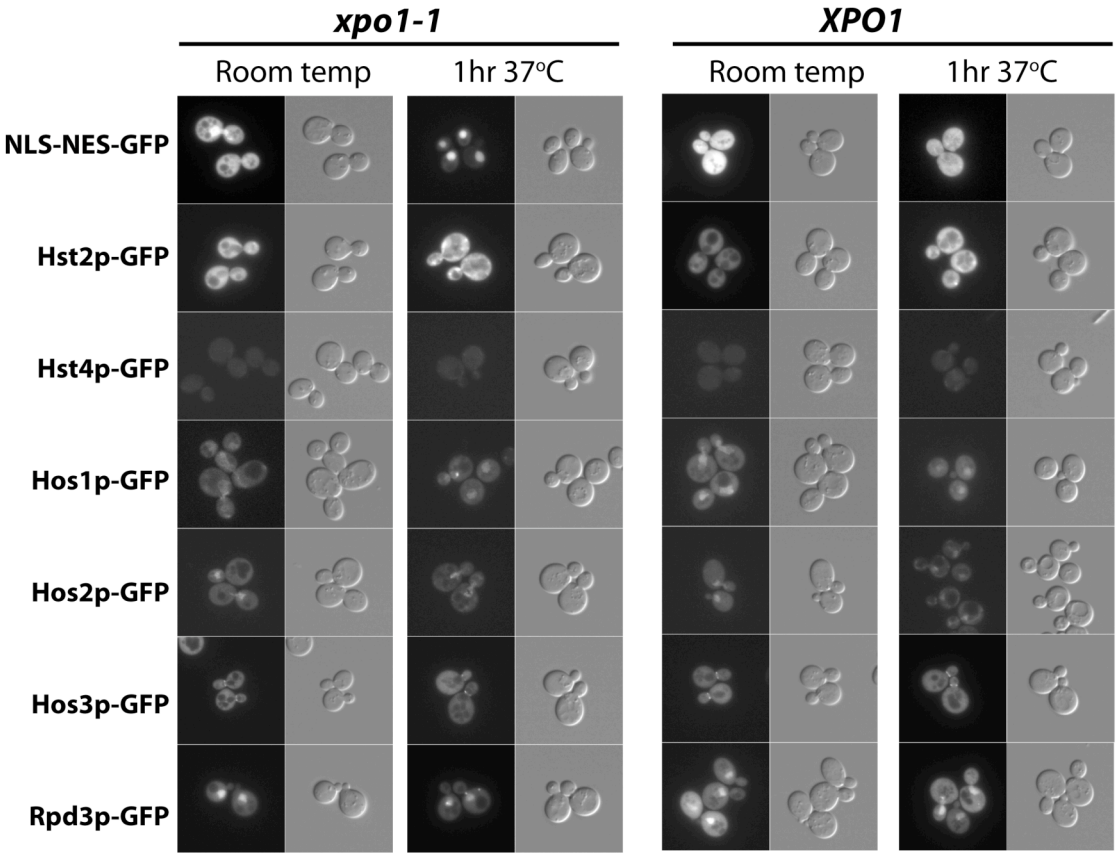


Figure 6.6. Analyis of deacetylases for *XPO1*-dependent nuclear-cytoplasmic shuttling. GFP-tagged deacetylases that have a cytoplasmic localization were expressed in wildtype (*XPO1*) and *xpo1-1* cells and grown in minimal media at room temperature. Localization was assessed after growth at permissive temperature (room temperature) and restrictive temperature (37°C) for one hour. NLS-NES-GFP (nuclear localization sequence – nuclear export sequence) is used as a positive control for a protein that is known to shuttle between the nucleus and cytoplasm. The corresponding DIC images are shown.

Nuclear-cytoplasmic shuttling properties of deacetylases via the CRM1 family shuttling pathway

The steady-state localization studies indicated that deacetylases are found both in the nucleus and the cytoplasm. We wished to determine if any of the proteins that are found in the cytoplasm shuttle between the nucleus and cytoplasm. This might

indicate that the protein's function is in the nucleus but perhaps is localized in the cytoplasm to spatially regulate its function.

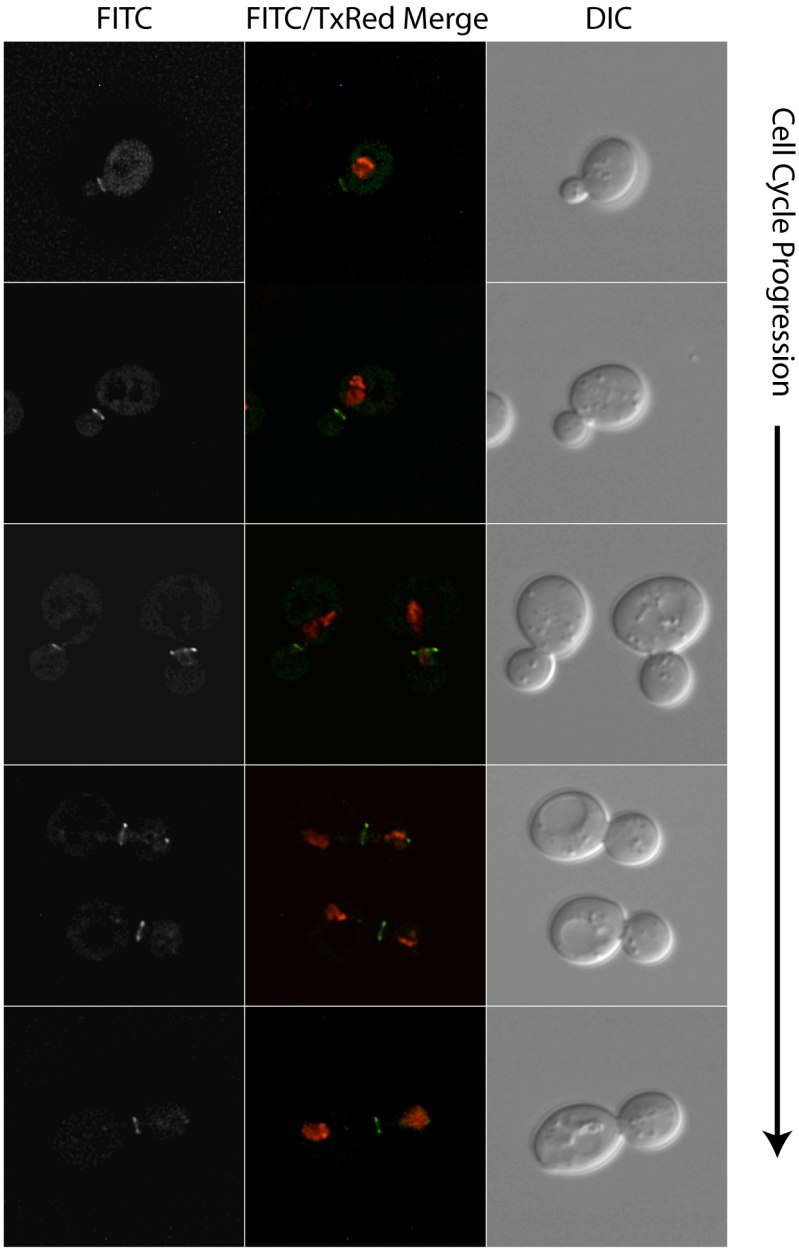
XPO1 encodes for a member of the CRM1 exportin family and the major nuclear exportin in yeast [11]. We used a temperature-sensitive mutant *xpo1-1* to determine if any deacetylases shuttle between the two compartments. When grown at permissive conditions, a shuttling protein would be found at its steady-state localization. However, when shifted to a restrictive temperature, 37^{o}C, the protein will concentrate in the nucleus because it will be imported into the nucleus but will be unable to be exported.

A control plasmid consisting of a nuclear localization sequence (NLS) and nuclear export sequence (NES) tagged with GFP undergoes nuclear-cytoplasmic shuttling (Figure 6.6). When expressed in cells grown at permissive conditions, the protein is both cytoplasmic and nuclear. However, when shifted to restrictive conditions for one hour, the protein accumulates in the nucleus. This same analysis using the cytoplasmic deacetylases suggests that these proteins do not undergo shuttling.

Hos3p localizes to the neck region and forms a collar similar to the septin proteins

Many proteins involved in the regulation of polarized exocytosis localize in a distinct manner to the sites of exocytosis (for example, see GFP-Sec4p localization in Figure 3.2). Exocytosis is a dynamic process where early in the cell cycle, vesicles are deposited at the daughter cell plasma membrane in a polarized fashion. Prior to cytokinesis, exocytosis is redirected towards the neck region between the two dividing cells. Steady-state localization of Hos3p-GFP is at the neck region. Therefore, we wanted to further investigate its localization.

Figure 6.7. Hos3p-GFP localization through the cell cycle. Hos3p-GFP was expressed in wildtype cells and viewed by fluorescence microscopy. Hos3p-GFP localization was analyzed at various stages of the yeast cell cycle, using the nucleus (Rpb10p-RFP) and bud size as a measure of the stage of the cell cycle. The corresponding differential interference contrast image is also shown. The FITC and TxRed channel images display the maximum projection from a deconvolved z-series.


FITC
FITC/TxRed Merge
DIC
Cell Cycle Progression

Hos3p-GFP localization was examined at various stages through the cell cycle. Sec4p, the exocytic Rab GTPase and other exocytic regulators localize to the neck region just prior to cytokinesis. We found that Hos3p-GFP localizes to the region between the mother and daughter cell for the entirety of the cell cycle (Figure 6.7). In this analysis, cell cycle was monitored using daughter cell size and nuclear positioning with an RFP nuclear marker. Very early in the cell cycle, Hos3p-GFP is found as a band localizing to the region between the mother and daughter cell. Once the nucleus begins to migrate into the daughter cell, Hos3p-GFP remains at the neck but also appears to localize to a structure surrounding the nucleus (notice in bottom three FITC images in Figure 6.7). In addition to a faint structure surrounding the nucleus, Hos3p-GFP is generally found as a bright point at the top of the migrating nucleus. This localization may indicate that Hos3p-GFP is concentrated at the daughter cell spindle pole in addition to being localized at the septin and in the cytosol. Colocalization analysis using known spindle pole markers will be required to confirm this localization. This may be an interesting aspect of Hos3p in polarity determination. In additions, these localization studies suggest that Hos3p-GFP may functionally impact processes occurring at the septin and also may impact processes at the spindle pole.

While both GFP-Sec4p and Hos3p-GFP localize to the mother-bud neck at times in the cell cycle, the nature of the localization appears to be slightly different. As illustrated in Figure 6.8, GFP-Sec4p is found as two ring-like structures at the mother-daughter interface in cells undergoing cytokinesis. Alternatively, Hos3p-GFP localizes as a single ring at the interface.

Similar to Hos3p-GFP, septins localize to the neck for the entire cell cycle [12]. These proteins form a filamentous collar around the neck and serve as a protein scaffold and physical barrier to compartmentalize the cell [13-15]. Therefore, we were interested in determining if Hos3p-GFP forms a collar or if it is continuous

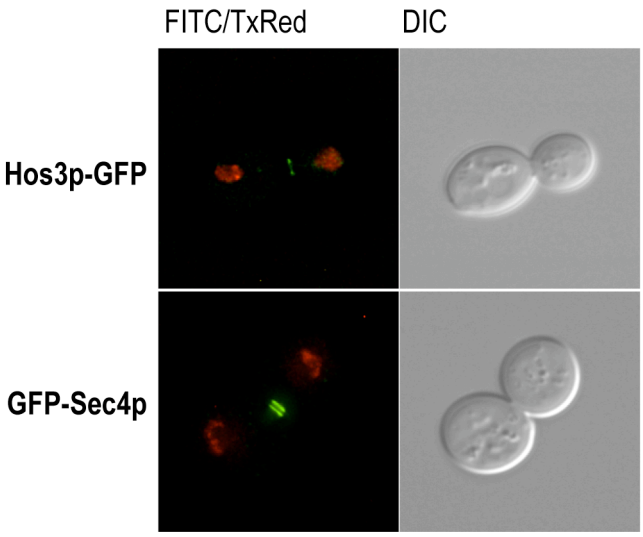


Figure 6.8. Hos3p-GFP and GFP-Sec4p localization at cytokinesis. Hos3p-GFP and GFP-Sec4p were expressed in wildtype cells and viewed by fluorescence microscopy. Localization was analyzed at cytokinesis, using the nucleus (Rpb10p RFP) and bud size as a measure of the stage of the cell cycle. The corresponding differential interference contrast image is also shown. The FITC and TxRed channel images display the maximum projection from a deconvolved z-series.

across the neck. Figure 6.9 shows a series of images through the depth of the cell at 0.4μm interval in the z-direction. When the center of the cell is imaged, Hos3p-GFP is localized to two bright points at the surface of the neck, suggesting that Hos3p does in fact form a collar around the neck in a similar manner to the septins.

Reconstruction of the z-series into a 3-dimensional image further verifies this finding (Figure 6.10). When rotated about the z-axis or tilted along the x-axis, a hole is found in the center of the fluorescent signal. Therefore, Hos3p-GFP localizes to the neck between the mother and daughter cell for the entire cell cycle and forms a collar around this region like the septin proteins.

Due to the similarity between the localization of Hos3p and the septin proteins, we wished to determine if Hos3p localization is dependent on an intact septin. This was analyzed using a *cdc12-6* temperature sensitive mutant, where growth at

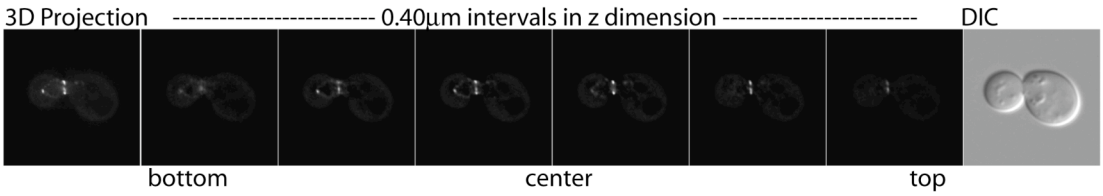


Figure 6.9. Hos3p-GFP localization through the cell depth. Hos3p-GFP was analyzed using a z-series with 0.2µm between each image. The z-series was deconvolved and a deconvolved slice is shown at 0.4µm intervals through the depth of the cell. The corresponding DIC image is shown.

restrictive temperatures causes aberrant septa and leads to defects in cytokinesis [16]. Growth at restrictive temperature for one or more hours, leads to a disruption of Hos3p-GFP localization at the neck between the mother and daughter cell where it is solely cytosolic (Figure 6.11). Therefore, Hos3p-GFP requires the septin for proper localization. This is not surprising as the septin is known to serve as a scaffold for many proteins at the mother-daughter cell interface.

Interestingly, upon disruption of the septin for three hours, where Hos3p-GFP no longer localizes to the neck, it can be found in a subset of the population localizing to a bright spot at what might be the spindle pole (Figure 6.11c). This was not observed at earlier time points and therefore the connection between the septin localization and the potential spindle pole localization requires further analysis. This observation will require further investigation using colocalization analysis, but suggests that Hos3p-GFP potentially localizes to the spindle pole independently of its septin localization.

Discussion

Deacetylase enzymes are one of the two classes of enzymes that regulate the cyclical acetylation modifications. Our analysis identified that deacetylases are found

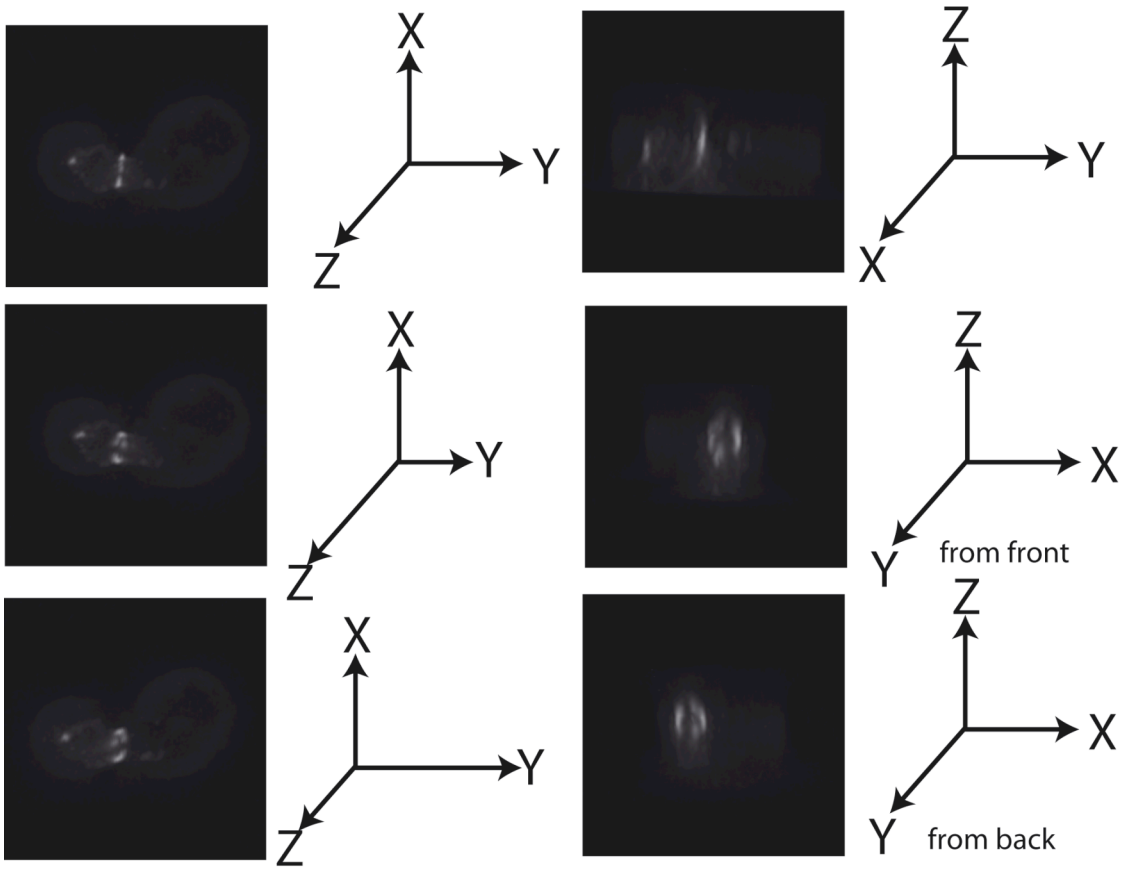


Figure 6.10. Hos3p-GFP forms a collar around the neck of the cell. A three dimensional reconstruction was done using the Imaris program of the deconvolved z series collected in Figure 6.9. The image is rotated about each axis (as indicated) to display the ring-like fluorescent structure.

in the nucleus and cytoplasm at steady state. Nuclear cytoplasmic shuttling experiments suggest that the deacetylases found in the cytoplasm do not undergo shuttling under the conditions that were tested for. Our results could be explained through a few models. The first is that the proteins do not shuttle between the two compartments. Therefore the proteins that are found both cytoplasmically and nuclear may be in two populations: a nuclear pool and a cytoplasmic pool that are not interchangeable. Another possibility is that these proteins use an alternative exportin

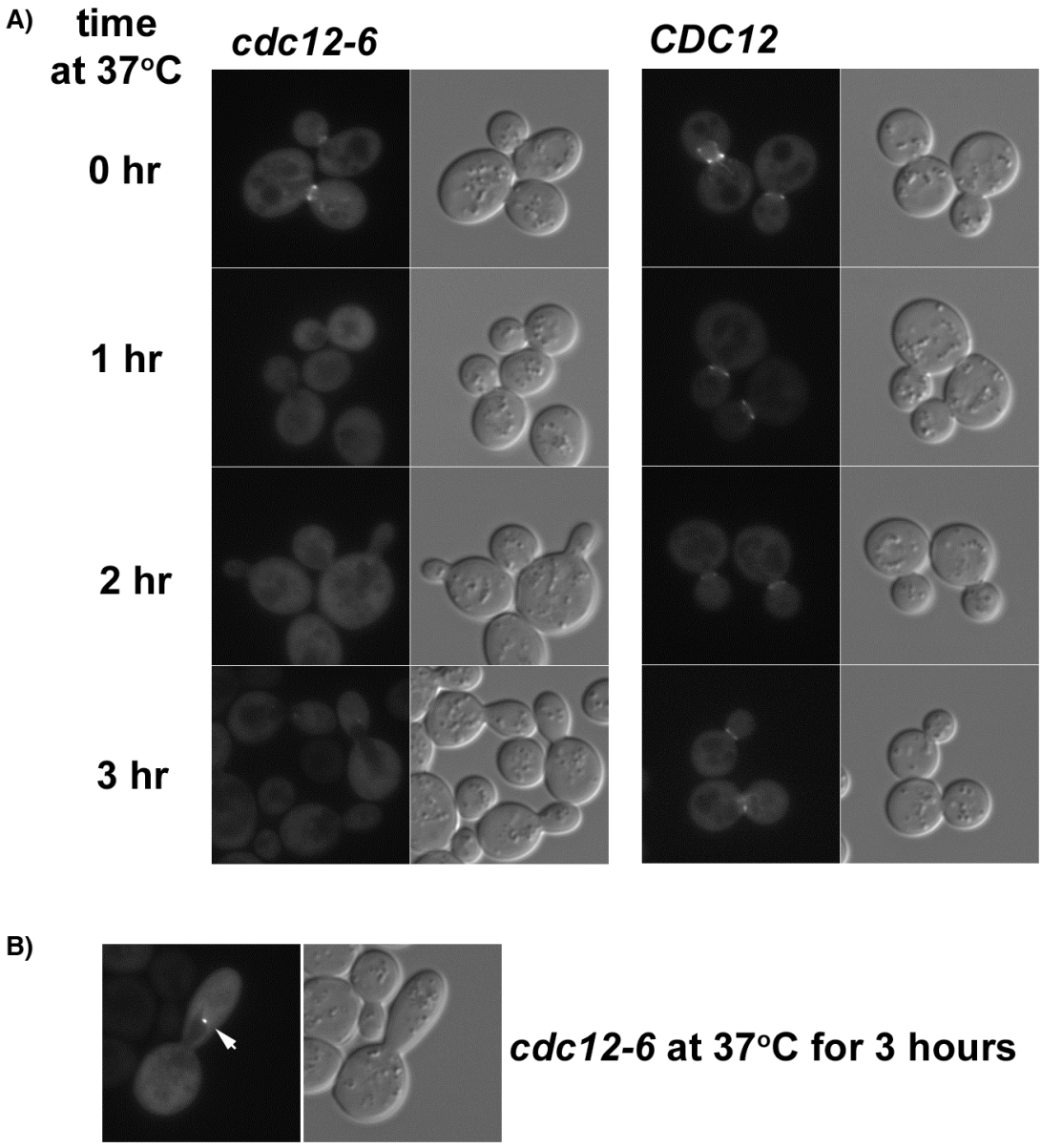


Figure 6.11. Hos3p-GFP requires the septin to maintain neck localization. A) Hos3p-GFP was expressed in wildtype (*CDC12*) and *cdc12-6* temperature sensitive mutants. Localization was assessed in minimal media after growth at restrictive temperature (37^{o}C) for the indicated time. B) Hos3p-GFP localization in *cdc12-6* cells after growth at 37^{o}C for three hours. This localization was observed in a subset of the population.

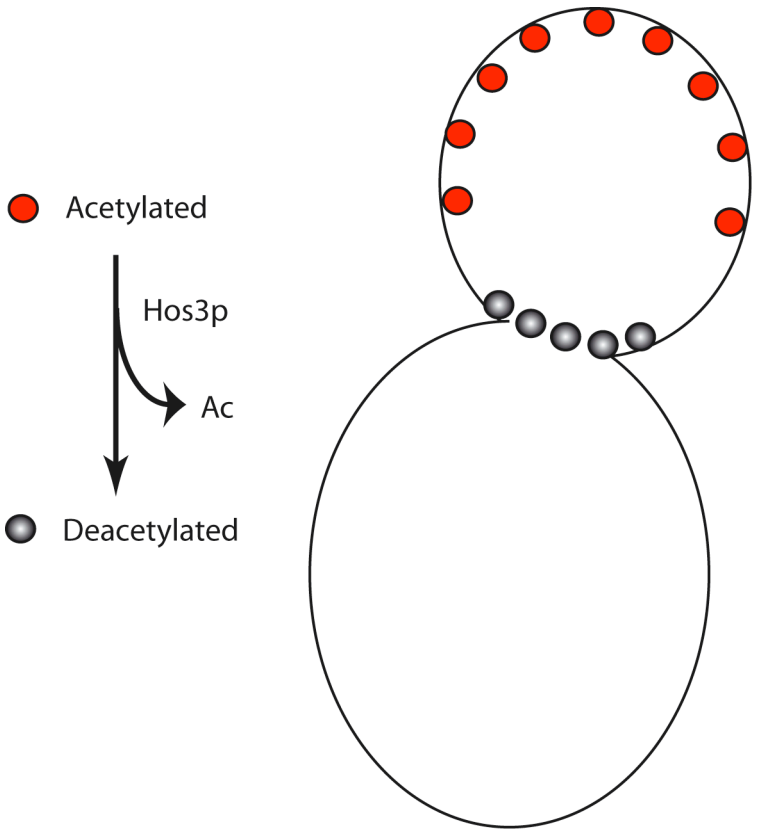


Figure 6.12. Model for Hos3p deacetylase activity at the neck to spatially regulate protein activity. Red circles represent potential Hos3p substrate when acetylated, blue circles represent potential Hos3p substrate without acetylation modification.

to shuttle from the nucleus to the cytoplasm. We analyzed each protein to determine if it shuttles using Xpo1p, the major exportin in these cells, but additional exportins exist [11]. Finally, these proteins may shuttle albeit with much slower kinetics than the NLS-NES-GFP control plasmid. Regardless of the nuclear cytoplasmic shuttling analysis, the steady state localization analysis demonstrates that similar to the Elongator acetyltransferase complex, deacetylases also localize to the cytoplasm. This implies that the cyclical regulation of acetylation also occurs in the cytoplasm as well as the nucleus to regulate protein function.

Hos3p appears to be the most intriguing deacetylase in the context of identifying a deacetylase whose regulation may impact exocytic events. Hos3p-GFP localizes to the neck in a similar manner to the septin proteins. In addition to septin proteins, the formin Bnr1p, which assembles actin cables, also localizes to the neck for the entirety of the cell cycle [17]. Septins and formins have functional impacts on the regulated secretion and cytokinesis [18-20]. An analysis using a septin mutant indicates that Hos3p requires an intact septin to maintain its localization. In this model, the septin may serve as a scaffold to maintain Hos3p to the neck. Perhaps Hos3p is responsible for the deacetylation of a factor, or factors, that impacts secretion. Our current model proposes that Hos3p deacetylation spatially regulates a protein's function to a particular region of the cell surface (Figure 6.12). Early in the cell cycle and similar to exocytosis, the protein function occurs towards the daughter cell periphery, however, prior to cytokinesis, it is redirected to the neck where Hos3p deacetylates the target protein to regulate protein function.

If Hos3p function is found to physiologically impact exocytosis through *in vivo* functional analysis, this protein could prove to be beneficial to further aid in the identification of acetylation targets of Elp3p or other substrates for acetylation that are involved in regulating secretion.

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Chapter 7. Urmylation is a ubiquitin-like modification involved in secretion regulation

Abstract

Urm1p is an evolutionarily conserved ubiquitin-like posttranslational modification whose function remains unclear. Studies in *S. cerevisae* have suggested that its function is linked to budding, invasive growth and sensing and controlling intracellular amino acid levels [1, 2]. Studies have linked urmylation to Elongator function, which led us to investigate the role urmylation might play in regulating exocytosis [2, 3]. Loss of Elongator function suppresses mutations in exocytic machinery [4]. This study presents data to suggest that the urmylation conjugation system also negatively regulates exocytosis as deletion of *URM1* or its E1 ligase *UBA4* suppresses mutations in the Rab guanine nucleotide exchange factor *SEC2*. The regulation of exocytosis by the Elongator complex and urmylation appear to be independent of their role in the TOR signaling pathway. Regulating exocytosis may be a way for urmylation to regulate amino acid levels in the cell through controlling the trafficking of specific amino acid transporters to the cell surface.

Introduction

Ubiquitin-like conjugation systems regulate many cellular functions

Covalent posttranslational modifications greatly increase the diversity of protein functions. Addition of small molecules, including phosphate, acetyl or methyl groups, to protein side chains can dramatically impact function. It is well established that polypeptides are also covalently attached to proteins and can modulate protein localization and function. Ubiquitin is the best characterized polypeptide conjugation system that targets proteins for degradation, endocytosis and other processes,

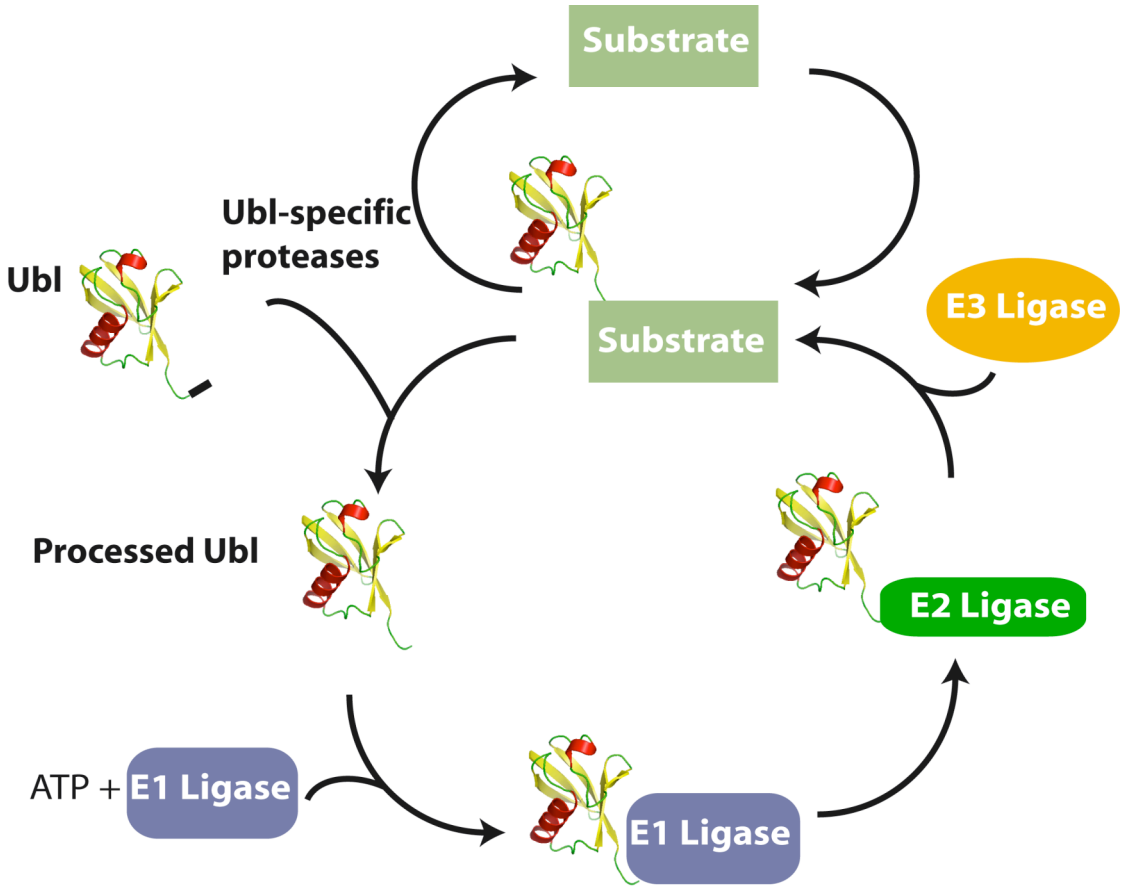


Figure 7.1. The ubiquitin conjugation cycle. The attachment of ubiquitin and other Ubls to its substrates generally requires the action of multiple enzymes. This figure illustrates the cycle the Ubl undergoes, where initially the pro-protein is processed to expose the C-terminal glycine. The processed Ubl is then conjugated to an E1, which can transfer it to an E2. Once conjugated to an E2, and E3 facilitates the attachment to the substrate. Two classes of E3 ligases are known for ubiquitin, where one class solely recruits the substrate for conjugation directly from the E2. The second class receives the conjugation from the E2 and then transfers ubiquitin to the proper target. Not all Ubl conjugation systems necessarily require the action of E1, E2 and E3 ligases as these enzymes are thought to allow for increased diversity in the conjugation targets. Ubl-specific proteases then catalyze the removal of the Ubl from target proteins, demonstrating the reversible nature of the polypeptide modifications.

Table 7.1. Ubiquitin-like modifications and their proposed functions

	Known Conjugating Enzymes	Known Cellular Function
Ubiquitin	E1, E2, E3	endocytosis, degradation, protein interactions, other
SUMO	E1, E2, E3	nuclear transport, transcription
Nedd8/Rub1	E1, E2, E3	meiosis to mitosis transition
ISG15	E1, E2, E3	immune response
Apg8	E1, E2	autophagy, vesicular transport
Apg12	E1, E2	autophagy, ctoplasm to vacuole targeting
Urm1	E1	budding, oxidative-stress response
UFM1	E1, E2	unknown
Fat10	---	apoptosis
Hub1	---	cell polarity

depending on the nature of the ubiquitin modification [5]. A family of ubiquitin-like proteins (Ubls) has been identified since the discovery of ubiquitin function. These proteins generally adopt a similar three-dimensional topology, although they do not necessarily display a high level of primary sequence identity, and utilize a C-terminal glycine residue as the site of attachment. Lysine residues on the substrate are the most common site of modification, where an amide bond forms between Ubl and the lysine side chain [5, 6].

Ubls are attached to substrates through a conjugation system, generally involving four enzymes (Figure 7.1) [5, 6]. Ubl-specific proteases (ULPs) modify the Ubl at its C-terminus to expose the gylcine carboxylate that is used for attachment. E1 enzymes use ATP to adenylate the Ubl and this forms a thioester bond between the Ubl and the E1 at its active-site cysteine. The Ubl is transferred from the E1 to an E2 active-site cysteine. The E2 attaches the Ubl to its substrate with the help of an E3

enzyme. For ubiquitin, two types of E3s are known. RING (*r*eally *i*nteresting *n*ew *g*ene) E3s generally act as adaptors and recruit the substrate and the ubiquitin-E2 and position two species to allow for transfer to the substrate. HECT (*h*omologous to *E*6-AP *C*-*t*erminus) E3s first receive ubiquitin from the E2 and then transfer ubiquitin to its substrate.

A number of Ubl modifiers have been identified in eukaryotes and have been demonstrated to regulate a diverse array of cellular functions (Table 6.1). SUMO (*S*mall *u*biquitin-related *mo*difier) is the best-characterized Ubl to date and has been shown to regulate chromatin structure, nuclear transport and translation [6]. Ubiquitin, SUMO, Nedd8 and ISG15 utilize E1, E2 and E3 enzymes to conjugate to proteins, however, it is unclear if E2 and E3 activities are critical for conjugation of all Ubls as E3 enzymes have not been identified for APG8, APG12 and UFM1 and neither E2 nor E3 enzymes have been identified for Urm1 [5, 7]. It is well established, however, that E2 and E3 enzymes can add to the diversity of proteins that receive polypeptide conjugates and the cellular functions that are affected by the particular conjugated system.

Urmylation is a conserved Ubl whose function is not well understood

Urm1 was identified as a Ubl with sequence homology to the *E.coli* ubiquitin-like conjugation systems Moa and ThiS (20% and 23% identity respectively). A Urm1 human counterpart was also identified, that is 42% identical, indicating a basic function in all eukaryotes [7]. Urm1p adopts a three-dimensional structure similar to ubiquitin and MoaD, demonstrating structural homology between these proteins (Figure 7.2) [8-10]. Uba4 has E1 enzymatic activity however the identity or requirement of E2 or E3 enzymatic activity has yet to be determined [7].

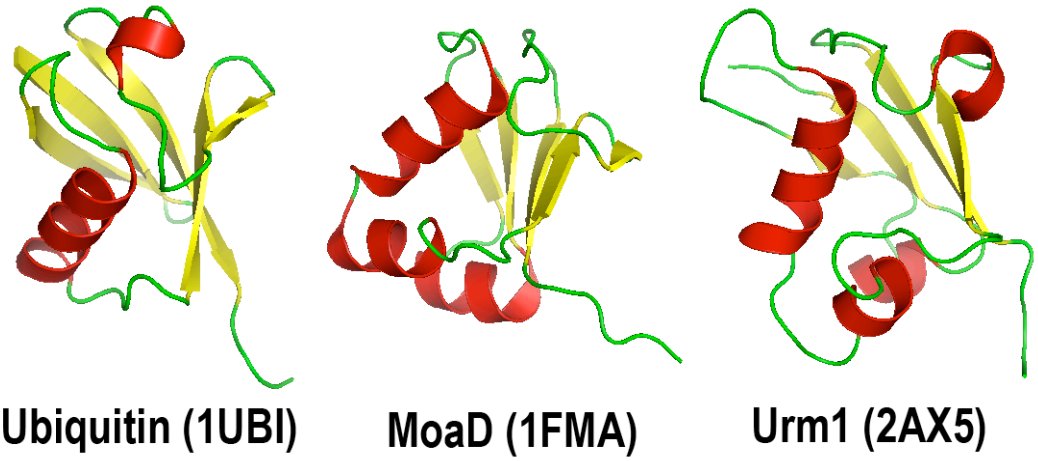


Figure 7.2. Structural conservation between Urm1p and other Ubls. Three dimensional structures of ubiquitin (1UBI), the bacterial Ubl MoaB (1FMA) and Urm1p (2AX5) highlighting the structural conservation between the three proteins [8-10]. Ubiquitin and MoaB structures were solved using x-ray crystallography and Urm1p structure was solved using NMR. The average conformation is shown. Alpha helices are shown in red, beta sheets are shown in yellow and loops are shown in green.

The *in vivo* function(s) of urmylation is not well understood. *URM1* and *UBA4* are required for cell survival when *CLA4* is deleted from cells, a p21-activated kinase involved in budding [2]. Furthermore, deletion of *URM1* and *UBA4* results in sensitivity to growth in the presence of rapamycin. Therefore urmylation has been suggested to be involved in regulating budding and nutrient sensing in yeast [1, 2]. Studies have demonstrated that multiple proteins are conjugated by Urm1p, however only one substrate, Ahp1p, has been identified [2, 7, 11]. Ahp1p is an antioxidant protein that is conjugated with Urm1p. This protein is not involved in budding but may play a role in nutrient sensing as deletion also confers sensitivity to rapamycin [2].

Elongator subunits *ELP2* and *ELP6* were also found to be essential for cell survival in a *cla4Δ* background. Loss of these two proteins also affects the level of at least one Urm1p conjugate [2]. Urmylation was further linked to Elongator function

by a study that found that Elp1p is proteolyzed at the N-terminus and urmylation enhances Elp1p proteolysis. The functional significance of proteolized Elp1p has not been addressed and it is unclear how urmylation regulates the proteolysis [3]. Our laboratory is interested in understanding the role of the Elongator complex as a negative regulator of exocytosis. This led us to investigate if the urmylation conjugation system is involved in regulating secretion and to try to determine how its function affects cell growth. Deletion of *urm1* and *uba4* suppresses the temperature sensitivity of *sec2* mutants indicating urmylation is involved in the regulation of secretion, further implicating the role of Urm1p in regulating budding [2]. As stated above, the cellular function(s) of urmylation have yet to be determined. We have developed a protocol to identify Urm1p-conjugates, which may provide insights into the pathways urmylation may impact.

Materials and methods

Strains and media

Yeast strains used in this study are listed in Table 3.2. Plasmids used in this study are listed in Table 7.3. YPD (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), SD (0.1% yeast nitrogen base, 2% D-glucose, plus required nutrients), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture) and SPM (0.02% raffinose, 0.3% KOAc) media was used.

Table 7.2. Yeast strains used in this study:

Strain number	Genotype
RCY241	Mat α *ura3-52 leu2-3,112 elp1Δ::URA3*
RCY241	Mat **a** *ura3-52 leu2-3,112 elp1Δ::URA3*
RCY271	Mat **a** *ura3-52*
RCY3696a	Mat **a** *ura3-52 urm1Δ::KAN*
RCY3497	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 tor1Δ::KAN^R*
RCY274	Mat α *ura3-52 sec2-59*
RCY3045	Mat **a** *ura3-52 leu2-3,112 elp1Δ::KAN^R sec2-59*
RCY3537	Mat α *ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 elp1Δ::KAN^R*
RCY3701	Mat **a** *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 tor1Δ::KAN^R*
RCY275	Mat **a** *ura3-52 sec2-41*
RCY3641	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 urm1Δ::KAN^R*
RCY3650	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 uba4Δ::KAN^R*
RCY267	Mat α *ura3-52 his4-619 sec2-41*
RCY3697A	Mat **a** *ura3-52 uba41Δ::KAN^R*
RCY3750	Mat α *ura3 leu2 his3Δ0 met15Δ0 elp1Δ::URA3 tor1Δ::KAN^R*
RCY3714	Mat α *ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 elp1Δ::KAN^R urm1Δ::KAN^R*
RCY3862	Mat **a** *ura3-52 sec2-59 urm1Δ::KAN^R*

Genetic analysis

A haploid strain carrying the mutation of interest was crossed with a haploid deletion strain. Diploids were isolated using temperature or nutrient selection. If this was not possible, the two haploid strains were crossed on YPD at 25^{o}C for approximately 5 hours. Zygotes were isolated using a micromanipulator on a Zeiss Axiolab microscope and grown on YPD for 2-3 days. Diploids were grown in liquid YPD media to log phase, washed once with sterile water and transferred to SPM media for sporulation. Cells were grown for 5-6 days at room temperature and after sporulation, tetrads were separated into individual spores using a micromanipulator. Spores were grown on YPD 25^{o}C for approximately 3-4 days. Spores were then tested for genotype and phenotype.

Table 7.3. Plasmids used in this study:

Plasmid Number	Plasmid	Source
RCB3610a	*his$_6$-FLAG-URM1* pRS316	This study
RCB3625	*his$_6$-URM1*(Δ97-99) pET15b	This study

Purification of Urm1-conjugated proteins

Two liter cultures each of *urm1ΔKAN* + vector (RCY3696A + pRS316) and *urm1ΔKAN* + his$_6$-FLAG-*URM1* (RCY3696A + pRC3610a) were grown in SC-uracil media at 30^{o}C to roughly 0.85 OD_{600}. The following procedure was followed for both samples. Cells were harvested by centrifugation and resuspended in 10ml of SC-uracil + 5mM NEM and grown for 15 minutes at room temperature. Cells were reharvested by centrifugation and resuspended in double the volume of cold lysis buffer (50mM Tris pH8.0, 100mM NaCl, 1% Trixon-X 100) + 30mM NEM + protease inhibitors (1mM phenylmethylsulphonyl fluoride, 10ug/ml pepstatin, 1mM benzamidine). Cells were lysed with three passes through a French pressure machine at 15,000 psi. Lysates were cleared by centrifugation at 10,000 rpm for 8 minutes at 4^{o}C. The supernatant volume was isolated and brought to 50ml of total volume with lysis buffer + 30mM NEM + protease inhibitors.

Lyates are added to 200μl of NiNTA resin, prewashed in lysis buffer and incubated end over end for 1.5 hours at 4^{o}C. The sample was added to a column and flow through was passed over the column an additional time. The column was washed with 15ml of lysis buffer, followed by 15ml of lysis buffer + 20mM imidazole. Bound protein was eluted with 1.5ml of lysis buffer + 200mM imidazole. The elution fraction was added to 30μl of prewashed αFLAG (M2) agarose conjugated resin and incubated end over end for one hour. The resin was washed six times with 1.5ml of FLAG wash buffer (50mM Tris pH8.0, 100mM NaCl, 0.2% Trixon-X 100).

Bound protein is eluted using AcTEV protease cleavage. A TEV cleavage site was engineered in pRC3610a between the FLAG epitope and *URM1* open reading frame to allow for TEV elution. Protein was eluted with 5 units of AcTEV protease overnight at 16°C. Supernatant was added to 10µl of NiNTA resin for one hour at room temperature to remove AcTEV protease. The supernatant is collected and added to 4X sample buffer without reducing agent. Samples are incubated at 50°C for 10 minutes and then used for analysis by SDS-PAGE followed by silver staining to analyze total protein composition of eluate and Western blot analysis using an αUrm1p polyclonal antibody (1:1,000 dilution in TBST) to identify proteins that have the Urm1p-conjugate.

Results

Urmylation regulates exocytosis

Our laboratory demonstrated that Elp1p and the Elongator complex negatively regulates exocytosis when *ELP1* was identified as an extragenic suppressor of a mutant Rab guanine nucleotide exchange factor (GEF), *sec2* [4]. Reports have identified a potential link between Elongator function and the urmylation conjugation system [2, 3]. This led us to investigate if urmylation is also involved in the regulation of exocytosis. Deletion of *URM1* suppresses the temperature sensitivity of *sec2-59* (Figure 7.3). This is a conjugation-specific regulation as deletion of its E1, *UBA4*, also suppresses *sec2-59* (Figure 7.3). The suppression is not *sec2* allele-specific. Similar to *elp1Δ,* loss of the urmylation pathway can also suppress the temperature sensitivity of *sec2-41* (Figure 7.4).

Loss of Elongator function and the urmylation conjugation system both partially suppress the secretion block generated by mutation of the Rab GEF *SEC2* [4]. We wanted to further investigate the *in vivo* relationship between the Elongator

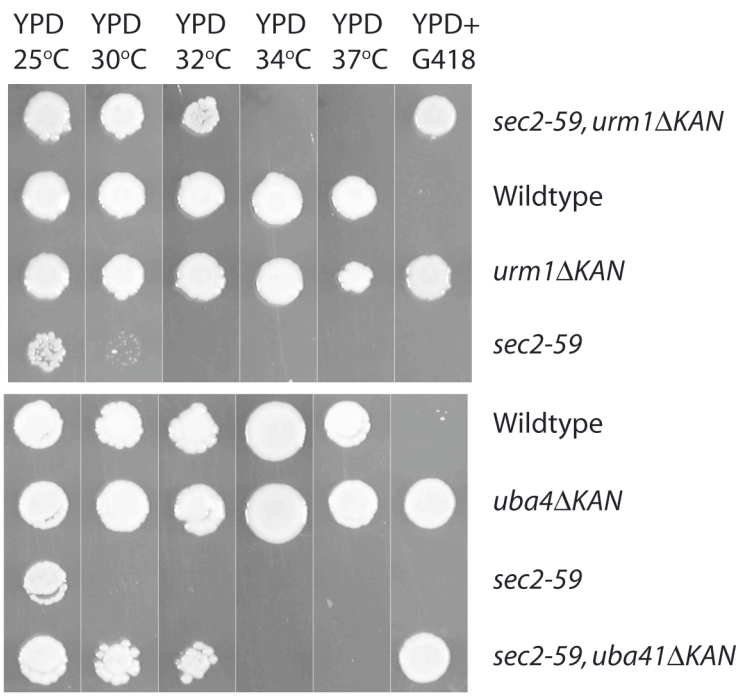


Figure 7.3. ***URM1* and *UBA4* negatively regulate exocytosis**. Strains containing *urm1Δ* or *uba4Δ* were crossed with a mutant strain containing *sec2-59*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

complex and the urmylation pathway. Both Elongator and urmylation function are nonessential under normal growth conditions [7, 12]. Cells containing a double deletion of *elp1* and *urm1* are viable however display a weak genetic interaction where the double mutant is temperature sensitive for growth on YPD at 40°C, where each single mutant is viable at this condition (Figure 7.5a). Cells lacking Elongator function are sensitive to growth on YPD with 2mM caffeine at high temperature (40°C and above) [13]. Cells lacking the urmylation pathway are not sensitive to caffeine, however, loss of both urmylation and Elongator function increases the temperature sensitivity when grown on YPD + 2mM caffeine, further suggesting an *in vivo* relationship between these two protein functions (Figure 7.5b).

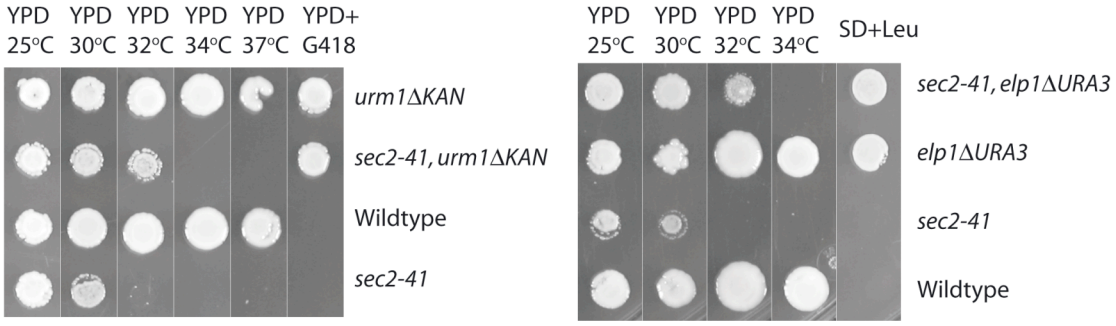


Figure 7.4. ***URM1* and *ELP1* suppression is not *SEC2* allele-specific**. Strains containing *urm1Δ* or *elp1Δ* were crossed with a mutant strain containing *sec2-41*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

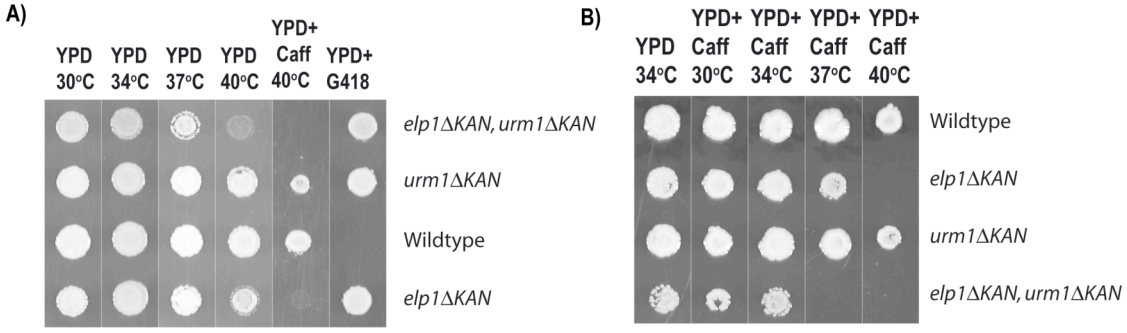


Figure 7.5. Genetic analysis of *URM1* and *ELP1*. A) A strain containing *urm1Δ* was crossed with an *elp1Δ* strain. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown. B) Isogenic strains isolated from tetrads were tested for growth on 2mM caffeine at varying temperatures. Cells were grown for 3 days.

These results led us to ask if Elongator requires the urmylation pathway to negatively regulate exocytosis or vice versa. Figure 7.6 illustrates that *sec2-59* cells are able to grow at 25°C but are temperature sensitive and unable to grow at 30°C and higher. Deletion of *ELP1* or *URM1* suppresses the temperature sensitivity and the cells can now grow up to 32°C, however remain temperature sensitive at higher

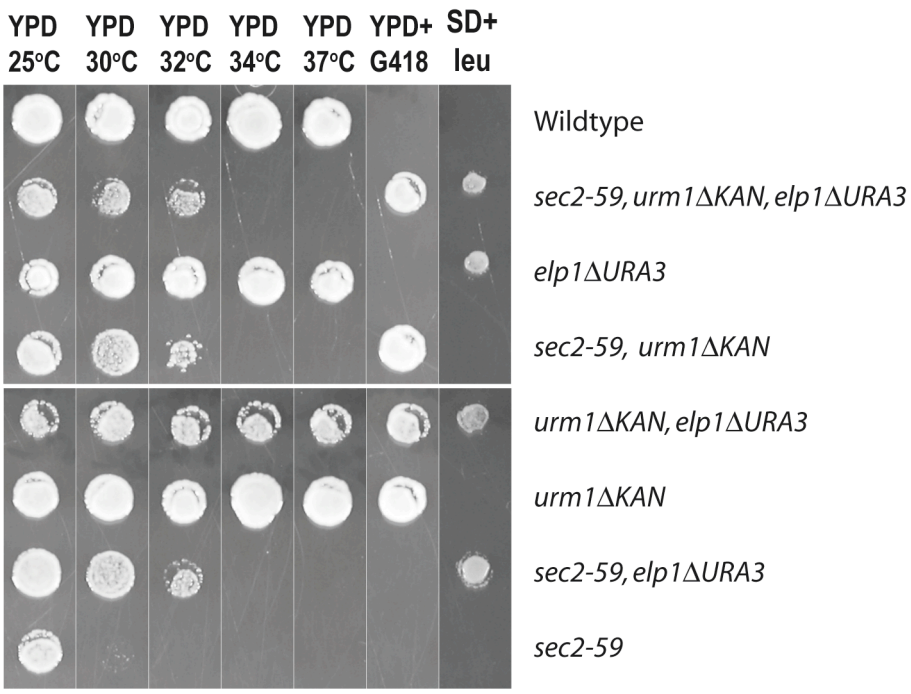


Figure 7.6. ***URM1* and *ELP1* negatively regulate exocytosis independently of the other**. A strain containing *urm1ΔKAN*^{R} was crossed with a mutant strain containing *sec2-59 elp1ΔURA3*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown.

temperatures. Deletion of both *elp1Δ, urm1Δ* in the same cell suppresses *sec2-59* to the same level as each deletion on its own suggesting that *ELP1* does not require *URM1* to suppress *sec2* temperature sensitivity or vice versa (Figure 7.6).

Urmylation and Elongator negatively regulate exocytosis independently from its involvement in TOR signaling

The TOR (*T*arget *o*f *R*apamycin) pathway is an evolutionarily conserved signaling pathway that regulates cell growth in response to nutrients [14]. A role for the urmylation conjugation system in the TOR signaling pathway has been

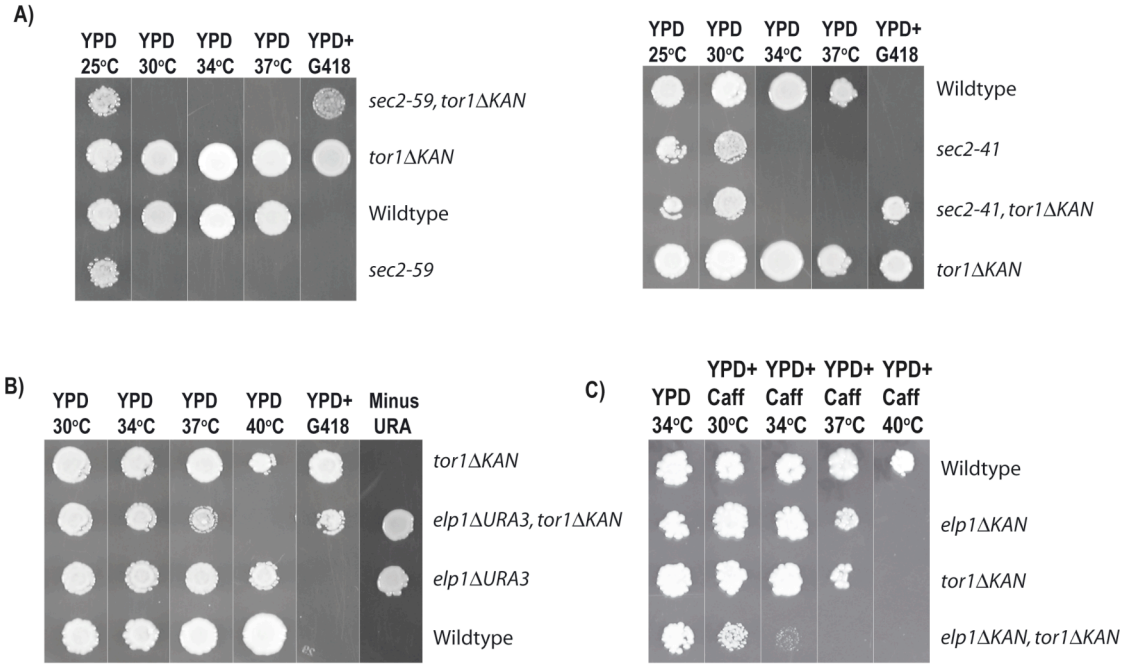


Figure 7.7. *TOR1* does not directly influence exocytosis. A) A strain containing *tor1Δ* was crossed with a mutant strain containing *sec2-59* or *sec2-41*. B) A strain containing *tor1ΔKAN*^{R} was crossed with *elp1ΔURA3*. Diploids were sporulated and tetrads were dissected to isolate individual spores. Genotypes and phenotypes of each spore from an isogenic tetrad were analyzed and a representative analysis is shown. C) Isogenic strains isolated from tetrads were tested for growth on 2mM caffeine at varying temperatures. Cells were grown for 3 days.

demonstrated, as loss of function results in hypersensitivity to growth on rapamcycin-containing media [1, 2].

Tor1p, the regulatory kinase in the TOR pathway, is a central regulator in nutrient sensing and regulating cell growth. Therefore, we were interested in determining if Tor1p influences the regulation of exocytosis in a similar manner to urmylation and Elongator. We found that deletion of *tor1* does not suppress temperature sensitive mutants of *sec2* (Figure 7.7a). Loss of Elongator function, through deletion of any subunit, does result in hypersensitivity to growth in the presence of rapamycin (data not shown) and a weak genetic interaction occurs

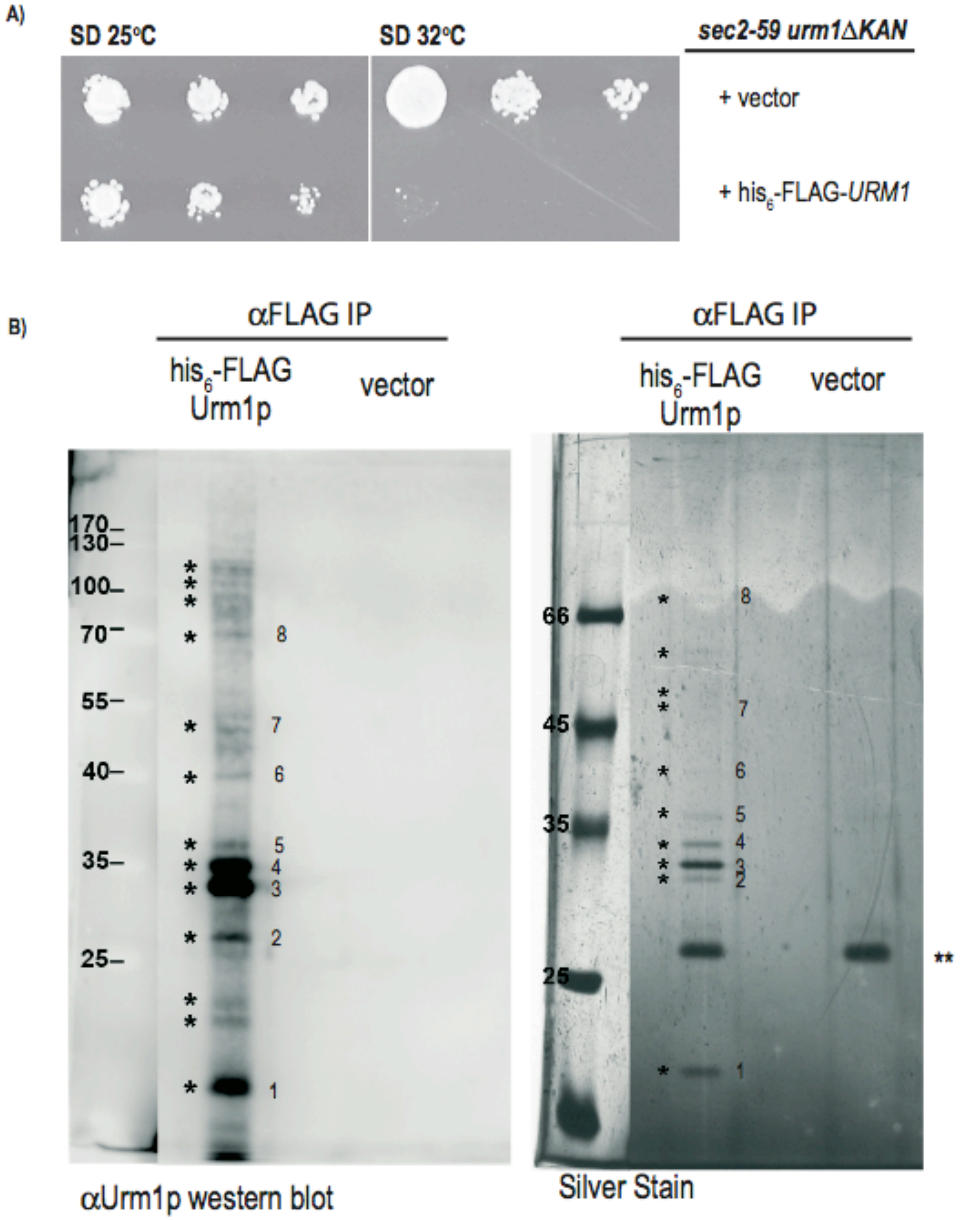
between *elp1* and *tor1* as a double deletion strain is temperature sensitive for growth on YPD 40^{o}C where each individual deletion is able to grow at the condition (Figure 7.7b). Furthermore, deletion of *tor1*, but not *tor2* (data not shown), causes sensitivity to 2mM caffeine at 40^{o}C (Figure 7.7b,c). Similar to what is seen in a double deletion strain of *urm1* and *elp1*, a double deletion strain of *tor1* and *elp1* has increased temperature sensitivity on YPD+2mM caffeine (Figure 7.7c).

These results suggest an *in vivo* role for the Elongator complex as a positive regulator in the TOR signaling pathway. The TOR signaling pathway does not appear to directly influence exocytosis in the same manner as Elongator and urmylation, as disrupting the TOR signaling pathway through deletion of the Tor1p kinase does not affect the temperature sensitivity of *sec2* mutants. Therefore, Elongator may be involved in the TOR signaling pathway downstream of its role in exocytosis.

Urm1-conjugated proteins

As discussed earlier, the cellular role for urmylation is not well understood. Genetic evidence indicates it regulates exocytosis but the mechanism of this regulation is unclear. Therefore we set out to determine its cellular function by identifying proteins that are conjugated with the Urm1p polypeptide. Identifying the targets of Urm1 conjugation will help elucidate the mechanism for how Urm1p negatively regulates exocytosis. We have begun developing a protocol to purify Urm1-conjugated proteins using a dual purification step. This protocol utilizes a strain where his$_6$-FLAG-Urm1p is the sole copy of Urm1p. This tagged version of Urm1p is able to negatively regulate exocytosis as adding it to *sec2-59 urm1ΔKAN* restores the thermosensitivity of these cells (Figure 7.8a). Therefore, one or more of the proteins conjugated with his$_6$-FLAG-Urm1p is responsible for the negative regulation of exocytosis.

Figure 7.8. Purification of Urm1p-conjugated proteins. A) Vector only or his_6-FLAG-Urm1p was transformed into *sec2-59 urm1*Δ*KAN* cells. Transformants were tested for growth on minimal media at 25°C and 32°C. Functionality can be assessed through restoration of the temperature sensitivity caused by the *sec2-59* mutations. B) Elution of purified Urm1p-conjugated proteins using NiNTA affinity beads followed by αFLAG immunoprecipitation with TEV protease elution. his_6-FLAG-Urm1p or vector only constructs were transformed into a *urm1*Δ strain and used for the purification. Protein composition is analyzed using silver stain to analyze total protein present. Western blot analysis using αUrm1p polyclonal antibody detects the presence of a Urm1p conjugate. Asterisk indicates potential Urm1p-conjugate. Double asterisk indicates TEV protease.


A)
SD 25°C
SD 32°C
sec2-59 urm1ΔKAN
+ vector
+ his6-FLAG-URM1
B)
αFLAG IP
his6-FLAG Urm1p
vector
αFLAG IP
his6-FLAG Urm1p
vector
170
130
100
70
55
40
35
25
66
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35
25
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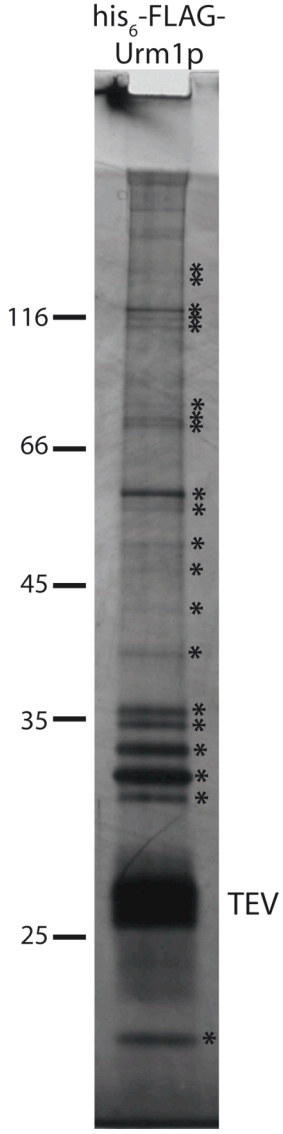
**
αUrm1p western blot
Silver Stain

Figure 7.9. Large scale purification of Urm1p-conjugated proteins. Elution of



purified Urm1p-conjugated proteins using NiNTA affinity beads followed by αFLAG immunoprecipitation with TEV protease elution. his_6-FLAG-in a *urm1*Δ strain was used for the purification. Protein composition is analyzed using silver stain to analyze total protein present. Asterisk indicates potential Urm1p-conjugate. TEV indicates TEV protease.

We have developed a protocol to purify Urm1p-conjugated proteins from large-scale cultures using NiNTA purification, followed by an αFLAG immunoprecipitation procedure. Protein composition was analyzed by silver stain to analyze total proteins present in the eluate and by Western blot, probing with an αUrm1p antibody to identify Urm1p-conjugated proteins (Figure 7.8b). From this analysis, it appears that a tandem purification protocol works well to generate a clean eluate containing primarily Urm1p-conjugate proteins, although identification of the less abundant conjugates may require larger cultures. Currently, this protocol allows us to purify roughly 16 to 18 Urm1p-conjugates from 2 liters of culture, grown to approximately 0.85 OD units (Figure 7.9). Additional scale up may be required to identify some of the high molecular weight proteins that appear to be present in low abundance.

Discussion

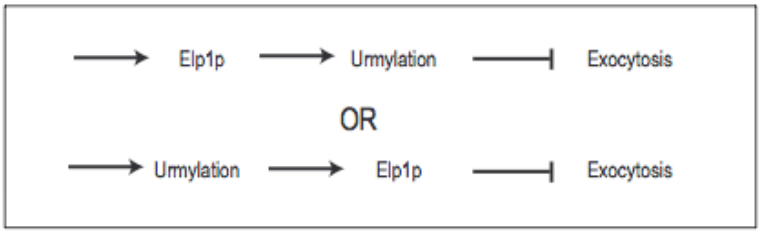
We found a role for the ubiquitin-like modification, Urm1p, in the regulation of exocytosis. Deletion of *urm1* or its E1 ligase, *uba4*, suppresses *sec2-59*, a mutant exocytic Rab exchange factor. The fact that *uba4Δ* also suppresses *sec2-59* indicates that the conjugation of Urm1p, and not unconjugated Urm1p, plays a role in this process. Therefore, we have begun to develop a protocol to purify and identify the proteins that are conjugated with Urm1p *in vivo*. The purification requires a tandem purification step that will be followed by mass spectrometry analysis for peptide identification.

Our interest in understanding the mechanism for how Elp1p and the Elongator complex negatively regulate exocytosis led to the identification of urmylation as being involved in this process. Loss of both Elongator function and the urmylation pathway suppress mutations in *SEC2*. However, deletion of both *elp1Δ, urm1Δ* in the same cell

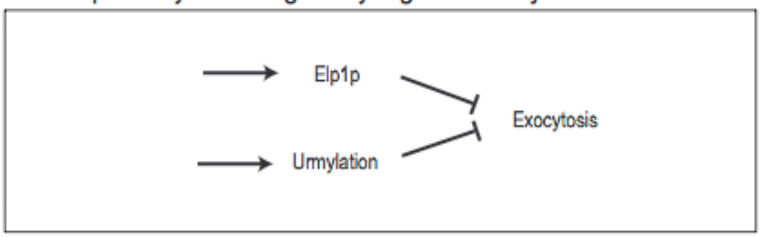
Figure 7.10. Models for regulation of exocytosis by the urmylation conjugation system. A) Model for how Elp1p and urmylation negatively regulate exocytosis either acting in the same pathway or in parallel pathways. B) Model for how urmylation may regulate intracellular amino acid levels through negatively regulating the transport of specific cargo to the cell surface.

A)

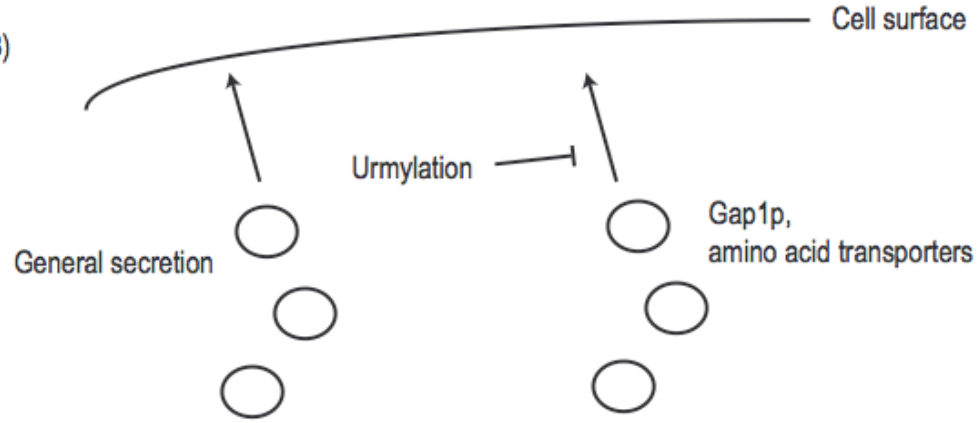
Same pathways with sequential function that negatively regulate exocytosis



Different pathways that negatively regulate exocytosis



B)



suppresses *sec2-59* to the same level as each deletion on its own, suggesting these two proteins may function sequentially in a pathway that negatively regulates exocytosis or function in two parallel pathways upstream of a point of convergence for regulation (Figure 7.10a). *ELP1* does not require *URM1* to suppress *sec2-59* and, similarly, *URM1* does not require *ELP1* for suppression. Other lines of evidence, discussed earlier, that indicate Elongator function and urmylation may be closely connected [2, 3].

Loss of Elongator function and urmylation cause hypersensitivity towards rapamycin, suggesting they positively regulate TOR signalling [1, 2]. The TOR pathway is involved in nutrient sensing and regulating cell proliferation in response to nutrients. Consistent with this, urmylation was also shown to be involved in sensing and regulating amino acid levels in cells [1].

Our identification of a role for urmylation in secretion regulation could provide insights into how urmylation is involved in sensing or regulating intracellular amino acid levels [1]. Exocytosis influences cell proliferation and growth as it provides membrane to the cell surface which is required for an increase in cell volume and surface area. In addition to lipids, plasma membrane proteins are also transported to the cell surface. Urmylation may negatively regulate exocytic events to regulate the transport of amino acid transporters to the cell surface in response to amino acid levels inside the cell. A specific protein would have a Urm1p-conjugation, which would negatively influence, in a regulated fashion, transport to the cell surface (Figure 7.10b). If amino acid levels drop in the cell, the negative regulation would be relieved through removal of the Urm1p-conjugation and more amino acid transporters would be transported to the cell surface to allow for increased amino acids flux across the plasma membrane.

Gap1p is a general amino acid transporter that displays a regulated trafficking pattern to the cell surface by the caffeine sensitive genes Gtr1p and Gtr2p (discussed in Chapter 5) [15]. A recent study found that urmylation also influences *GAP1* gene repression through Nil1p and Gln3p, where loss of the urmylation pathway results in a derepression of *GAP1* expression [1]. Immunofluorescence localization analysis suggests that Urm1p, and therefore, Urm1p conjugates localize to a punctate distribution in cells, suggesting that it functions in the cytoplasm and not the nucleus [2]. Therefore, urmylation may provide a feedback mechanism to control amino acid levels to control amino acid transporters through regulating gene expression and trafficking to the cell surface.

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Chapter 8. A new set of RFP markers for colocalization studies in *S.cerevisae*

Abstract

Fluorescence microscopy is a powerful technique to study organelle structure, however, it requires specific organelle markers for the analysis. A new set of RFP organelle markers are described here that localize to a many subcellular structures. Verification of intracellular localization is also presented through colocalization or mutational analysis. These markers will be beneficial for studying processes in live cells or fixed cells and will have multiple applications.

Introduction

Cells are a collection of complex four-dimensional networks of interactions among biosynthetic molecules. Many of the interactions in eukaryotic cells are spatially regulated by the elaborate set of intracellular organelles. These membrane-bound compartments generate microenvironments where particular proteins, lipids and nucleic acids are concentrated to create an environment for specific chemical reactions. For example, peroxisomes are enriched in enzymes that rid the cell of certain toxins.

Cells and their organelles are constantly undergoing changes in response to their environment. Biochemical and genetic techniques are often used to investigate specific changes in biosynthetic molecules while perturbing the system in a regulated fashion. An additional method used to monitor changes at the subcellular level includes the use of specific fluorescent markers in conjunction with microscopy. Fluorescence microscopy is a powerful technique where the localization of a molecule of interest is probed using a specific reporter. Examples of commonly used reporters are conjugated antibodies or genetically encoded reporters.

An antibody, conjugated with an excitable fluorophor that recognizes a specific protein, can identify the protein's subcellular localization. An advantage of this technique is that antibody-conjugated fluorophores tend to be very bright and stable, therefore, less prone to photobleaching over time. A drawback to this technique is that cells require fixing and permeabilization in order to introduce the antibody to the cell interior for antigen recognition.

The fluorescent reporter can also be genetically encoded, generating a fusion protein consisting of the protein of interest fused to a fluorescently excitable polypeptide. Green fluorescent protein (GFP) and red fluorescent protein (RFP) are two examples of genetically encoded localization reporters. An advantage of this technique is that a protein can be visualized in real time in live cells. A disadvantage of genetically encoding the reporter is that, as with the creation of any fusion protein, the tag may alter protein function.

The simple eukaryote *S.cerevisae* presents a powerful system to study cellular structure because of the conservation of many key features among eukaryotes, its ease of genetic manipulation, and the ability to study cellular processes *in vivo* over time using live cell imaging. Combining these features allows one to observe changes at the subcellular level, in real time, caused by perturbations, which can provide valuable functional insights and help probe a protein's function. Studying *S.cerevisae* at the organelle level can be challenging, however, as many of the organelles have a punctate distribution in cells, including the Golgi, peroxisomes and endosomes. For example, the Golgi apparatus has a punctate pattern and does not take on the characeteristic shape found in higher eukaryotes despite having a conservation of many Golgi resident proteins. Therefore, reliable markers are required to identify the specific organelles. GFP is the most commonly used genetically encoded localization reporter and therefore we wished to generate a set of RFP markers that could be used for

Table 8.1. Strains used in this study.

Strain Number	Genotype	Source
NY605	Mat **a** *ura3-52 leu2-3,112*	Novick Laboratory
RCY252	Mat **a** *ura3-52 leu2-3,112 sec6-4*	Novick Laboratory
RCY397	Mat **a** *ura3-52 leu2-3,112 dnm1ΔKAN^R*	This study
RCY2907	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0*	Collins Laboratory
RCY3766	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 pex17ΔKAN^R*	Research Genetics
RCY3767	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 pex5ΔKAN^R*	Research Genetics
RCY3768	Mat α *ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 pex7ΔKAN^R*	Research Genetics
RCY939	Mat **a** *ura3-52 leu2-3,112* GFP-YPT53::*URA3*	This study
RCY2415*	Mat **a**/α *ura3Δ0/ura3Δ0 leu2Δ0/leu2D0 his3Δ0/his3Δ0 lys2Δ0/LYS2 met15Δ0/MET15 vps21ΔKAN/vps21ΔKAN GFP-VPS21::URA3*	This study

* integration is 3' of *vps21* locus

colocalization studies with GFP-tagged proteins. This study describes a new set of RFP fusion proteins that can be used as subcellular markers for many applications including studying changes in *S.cerevisae* organelles in live cells.

Materials and Methods

Strains and media

Yeast strains used in this study are listed in Table 8.1. Plasmids used in this study are listed in Table 8.2. Cells were grown in YPD media (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), SCD-ura or SCD-leu (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), SD media (2% D-glucose, 0.1% yeast nitrogen base and required nutrients), or YNO media (0.67% yeast nitrogen base without amino acids, 0.1% yeast extract, 0.05% Tween 40 and 0.1% oleic acid).

Table 8.2. Plasmids used in this study.

Plasmid Number	Plasmid	Source
pRC1243	GFP-Ypt7p *pRS316*	Buvelot *et al.* 2006
pRC2098	GFP-Sec4p *pRS316*	Buvelot *et al.* 2006
pRC2125	RFP-Sec2p *pRS315*	This study
pRC2237A	Sec21p-RFP *pRS316*	This study
pRC2239A	Sec13p-RFP *pRS316*	This study
pRC2240A	RFP-Sec7p *pRS316*	Calero *et al.* 2003
pRC2252	RFP-Gos1p *pRS316*	This study
pRC2258	RFP-Sed5p *pRS316*	This study
pRC2601	RFP-Rpb10p *pRS315*	Rahl *et al.* 2005
pRC2668	Sfk1p-RFP *pRS315*	This study
pRC2715	RFP-Gal4BP *pRS316*	This study
pRC2781B	RFP-Snc2p *pRS315*	This study
pRC2792A	Snq2p-RFP *pRS315*	This study
pRC2864	Pdr16p-RFP *pRS315*	This study
pRC2865A	Sna2p-RFP *pRS315*	This study
pRC2866	Fat1p-RFP *pRS315*	This study
pRC2867	Faa4p-RFP *pRS315*	This study
pRC2868	Ypt35p-RFP *pRS315*	This study
pRC2869	Erg6p-RFP *pRS315*	This study
pRC2870C	Tvp23p-RFP *pRS315*	This study
pRC2883C	PTS1-RFP *pRS315*	This study
pRC2906B	Snx41p-RFP *pRS315*	This study
pRC2969	Vac14p-RFP *pRS315*	This study
pRC2970A	Ycf1p-RFP *pRS315*	This study
pRC2971	$Cpy_{(1-50)}$-RFP *pRS315*	This study
pRC2972A	Vtc1p-RFP *pRS315*	This study
pRC3075A	Bpt1p-RFP *pRS315*	This study
pRC3588A	RFP-KDEL *pRS315*	This study
pRC3579B	GFP-Rnt1p pRS316	This study
pRC3674A	Img1p-RFP pRS315	This study

Generation of RFP fusion proteins

RFP fusion proteins were generated in pRS315 or pRS316 shuttle plasmids. When RFP was fused with the open reading frame (ORF) at the N-terminus, it was fused in frame with an amino acid linker (SAGGGSASAGGPGG where S is serine, A is alanine, G is glycine and P is proline) between RFP and ORF. The protein's

endogenous promoter was used to control expression. When RFP was fused at the C-terminus, the linker above was engineered between the ORF and RFP with the *ADH1* terminator. Constructs were generated using the overlap PCR method in yeast. Linearized vector and the required PCR products were transformed into wildtype yeast. Transformants were selected for on minimal media. Growth on minimal media indicates that the linearized vector was recircularized using the added PCR products. Generally, ten independently generated constructs were tested for expression using live cell microscopy. Constructs that expressed were recovered from yeast using standard techniques and further analyzed by restriction enzyme digestion. Localization in the appropriate compartment was confirmed through the colocalization studies or mutant analysis, as described below.

Microscopy

Plasmids were transformed into NY605. Transformants were grown overnight at room temperature in SD media containing the required nutrients. Live cells were analyzed with a Nikon Eclipse E600 microscope, 100X (1.4NA) lens, 1X optavar (0.08μm/pixel), and imaged using a Sensicam EM High Performance camera (The Cook Corporation). Differential interference contrast (DIC) images were taken at one plane. Fluorescent images were collected using deconvolution microscopy with a 0.2μm z step size. RFP-tagged proteins were analyzed with a TxRed filter (excited at 540-580nm and emission at 600-660nm). GFP-tagged proteins were analyzed with a FITC filter (excitation at 465-495nm and emission at 515-555). Images were captured with IP Lab 3.6.5 software and blind deconvolution with 30 iterations was done using Autodeblur and Autovisualize 9.1 software.

To validate the peroxisome localization of PTS1-RFP, the plasmid was transformed into isogenic wildtype, pex17ΔKAN, pex5ΔKAN, and pex7ΔKAN cells.

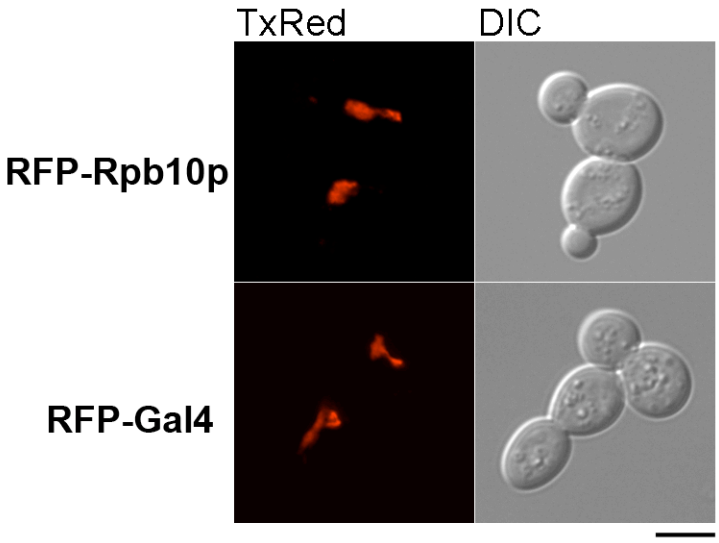


Figure 8.1. RFP nucleus maker. RFP nuclear markers expressed in wildtype cells. A deconvolved fluorescent slice from the center of the cell is displayed with the corresponding DIC image. Scale bar represents 5μm.

Transformants were grown in YNO media containing the required nutrients at room temperature for approximately 15 hours. Deconvolution microscopy was done following the protocol described above.

Colocalization studies with lipid droplet markers and bodipy green were done using 30μg/ml bodipy green for one hour at 30ºC. Cells were washed three times with PBS and then analyzed.

Three-dimensional reconstructions were done using the Imaris program using deconvolved z-series images processed using IP lab and Autodeblur as described above.

Results

Analysis and verification of RFP Markers

RFP-fusion proteins were created for yeast proteins localizing to various intracellular compartments. Fusion proteins localizing to the nucleus, daughter cell plasma membrane, sites of polarized exocytosis, mother cell plasma membrane, peroxisomes, endosomes, lipid droplets, vacuole, endoplasmic reticulum, and the Golgi apparatus were created on episomal plasmids that will be advantageous for studying processes in live cells.

Nuclear markers

RFP-tagged Gal4binding protein (Gal4BP) and RFP-Rpb10p, a transcriptional activator and subunit of RNA polymerase II, respectively, were created that generate a nuclear localized marker [1]. They can be seen in live cells as oval-like structures in cells early in the cell cycle or as dynamic dividing nuclear structures during nuclear division (Figure 8.1). This localization is consistent with the localization of Rbp10p-GFP that was generated in global analysis of protein localization in yeast [2]. These markers have proven to be reliable nuclear markers for our laboratory to determine the cell cycle stage of a particular cell.

Endoplasmic reticulum markers

RFP-KDEL construct was generated that localizes to the endoplasmic reticulum. KDEL is a signal sequence used for retention in the ER. RFP-KDEL is a luminal protein that localizes to both the nuclear and peripheral ER (Figure 8.2).

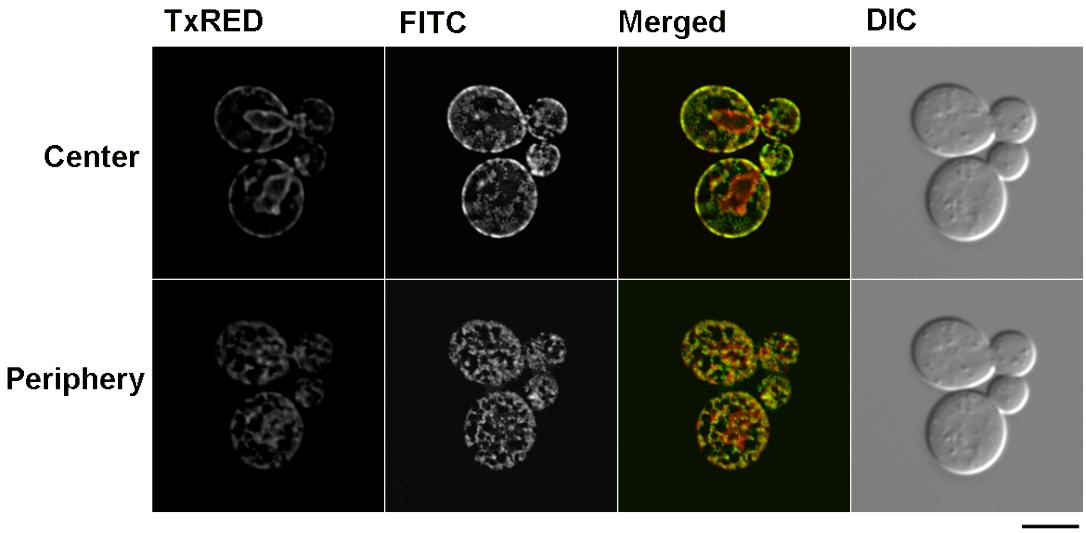


Figure 8.2. RFP-KDEL is an ER marker. RFP-KDEL and GFP-Rtn1p expressed in wildtype cells. A deconvolved fluorescent slice at the center and the cell periphery are displayed with the corresponding DIC image. Scale bar represents 5μm. Figure courtesy of David Cragun.

Golgi markers

RFP-Sec7p, RFP-Sed5p, Sec21p-RFP, DsRed-Gos1p, and Sec13p-RFP fusions localize to the Golgi apparatus (Figure 8.3). Sec7p is an Arf GTPase exchange factor that regulates vesicle budding from the Golgi [3]. Sed5p is a *cis* Golgi t-SNARE and Gos1p is a Golgi v-SNARE that function in vesicle transport [4, 5]. Sec21p and Sec13p are both components of vesicle coat complexes. Sec21p is a subunit of the COPI coat complex, the coat for retrograde traffic from the Golgi to the ER [6]. Sec13p is a component of the COPII coat complex found on ER to Golgi anterograde vesicles [7]. These fusion proteins have a punctate distribution throughout the cell. RFP-Sec7p localizes to both the mother cell and the daughter cell, but shows a slight polarization towards the daughter cell, suggesting that RFP-Sec7p marks regions of the *trans* Golgi. The other Golgi markers have a uniform distribution in both the mother cell and daughter cell. According to the protein localization database, Sec7p-

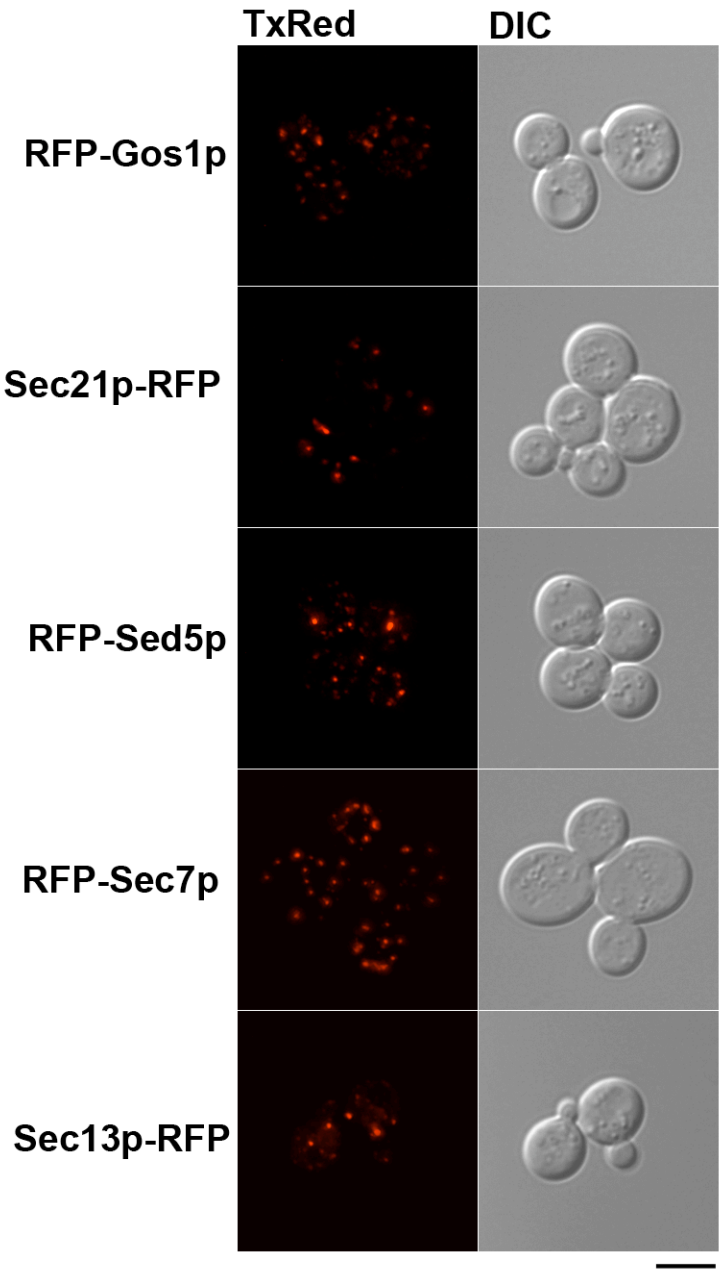


Figure 8.3. RFP Golgi markers. RFP Golgi markers expressed in wildtype cells. A deconvolved 2D maximum projection of a 3D z-series is shown with the corresponding DIC image. Scale bar represents 5μm.

GFP localizes to the late Golgi and Sec21p-GFP and Sec13p-GFP localize to ER to Golgi structures [2]. This is consistent with the localization of our RFP tagged fusion proteins. The localization of Gos1p-GFP and Sec5p-GFP were not analyzed in the localization database [2].

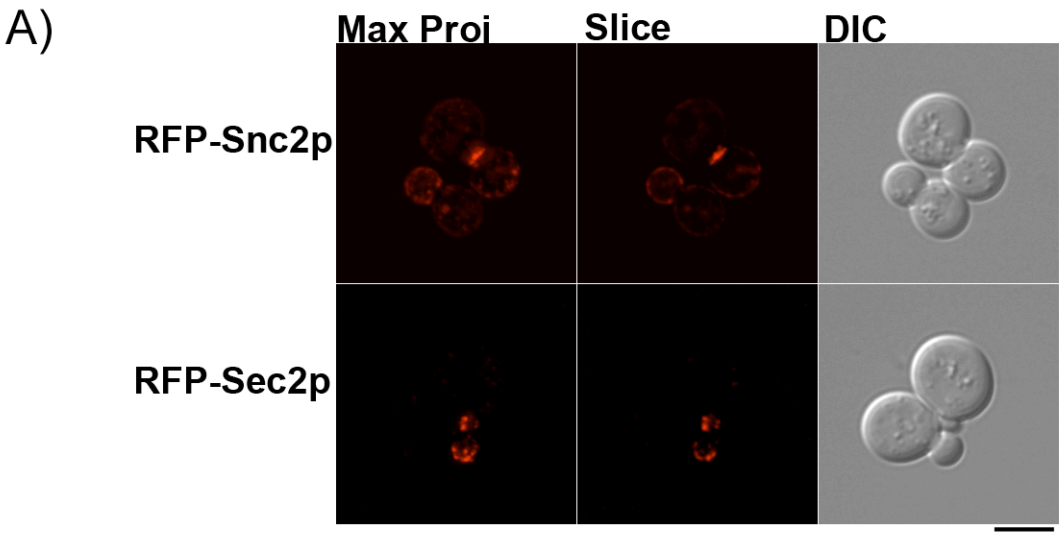
Exocytic markers

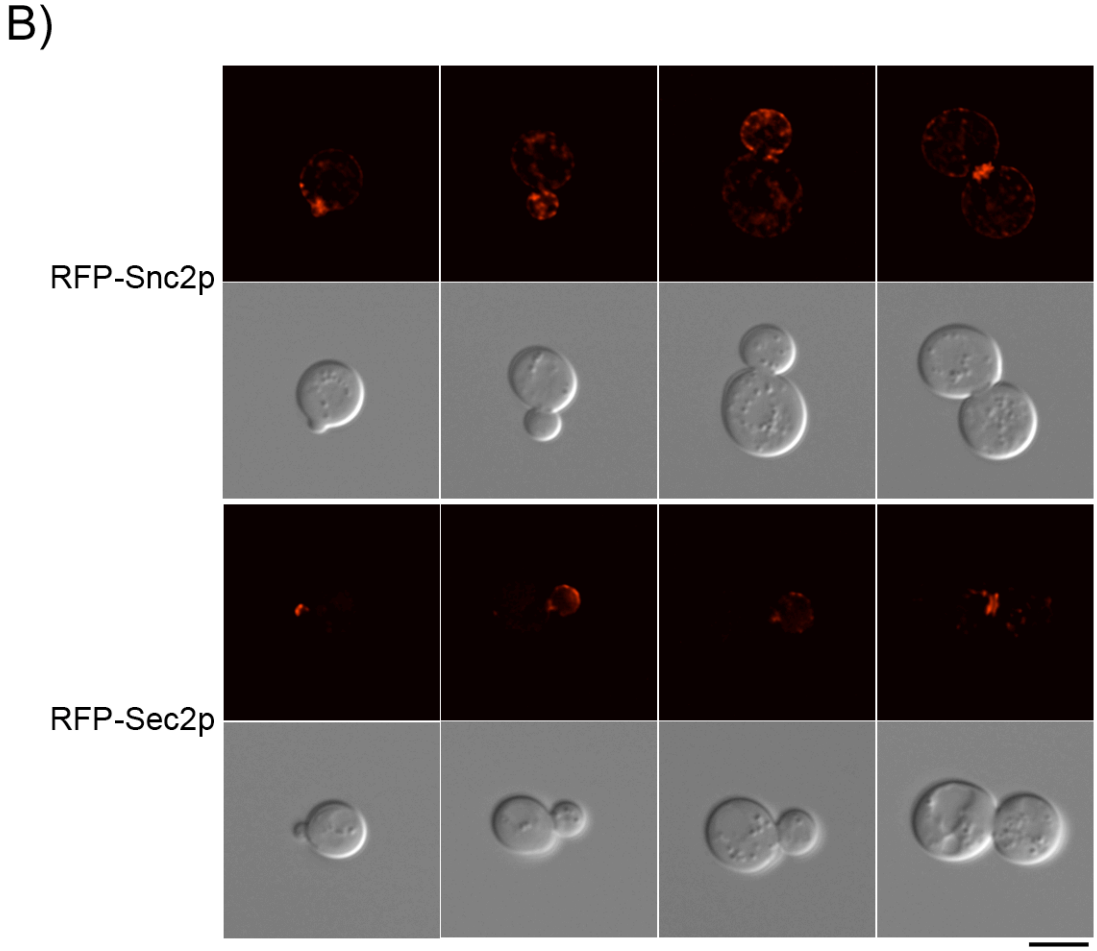
RFP-Snc2p was created that has a polarized distribution to the daughter cell in a cell cycle dependent manner (Figure 8.4). As cells progress through the cell cycle, RFP-Snc2p, a vesicle SNARE that functions in Golgi to plasma membrane trafficking [8], changes localization from the bud tip and daughter cell to the neck between the mother and daughter cell. RFP-Sec2p, a fusion protein of the Sec4p Rab guanine nucleotide exchange factor [9], localizes to post-Golgi vesicles and can be observed in a similar distribution as RFP-Snc2p (Figure 8.4). During early stages of the cell cycle, RFP-Sec2p localizes to the sites of polarized secretion in the daughter cell and as the cell progresses towards cytokinesis, RFP-Sec2p localizes to the neck (Figure 8.4 *bottom*). The localization of RFP-Sec2p is consistent with the protein localization database that used GFP-tagged versions of each protein [2]. The database determined the localization of Snc2p-GFP localizes to the vacuolar lumen. This is presumably due to the fact that Snc2p is tagged at its C-terminus for the database, which could interfere with function and localization. The localization of RFP-Snc2p is consistent with its function in Golgi to plasma membrane transport.

Plasma membrane markers

Snq2p-RFP and Sfk1p-RFP fusion proteins localize in a polarized distribution to the plasma membrane of the mother cell of the yeast (Figure 8.5). Snq2p-RFP is a transmembrane protein, an ABC transporter, which also localizes to the mother cell

Figure 8.4. RFP markers for sites of exocytosis. RFP exocytosis markers expressed in wildtype cells. (*top*) A 2D maximum projection of a deconvolved 3D z-series and a deconvolved slice are shown with the corresponding DIC image. (*bottom*) Fluorescent images of cells at different stages in the cell cycle were captured and deconvolved. Deconvolved slices from the cell center are shown with the corresponding DIC image. Scale bar represents 5μm.


A)
Max Proj
Slice
DIC
RFP-Snc2p
RFP-Sec2p


B)
RFP-Snc2p
RFP-Sec2p

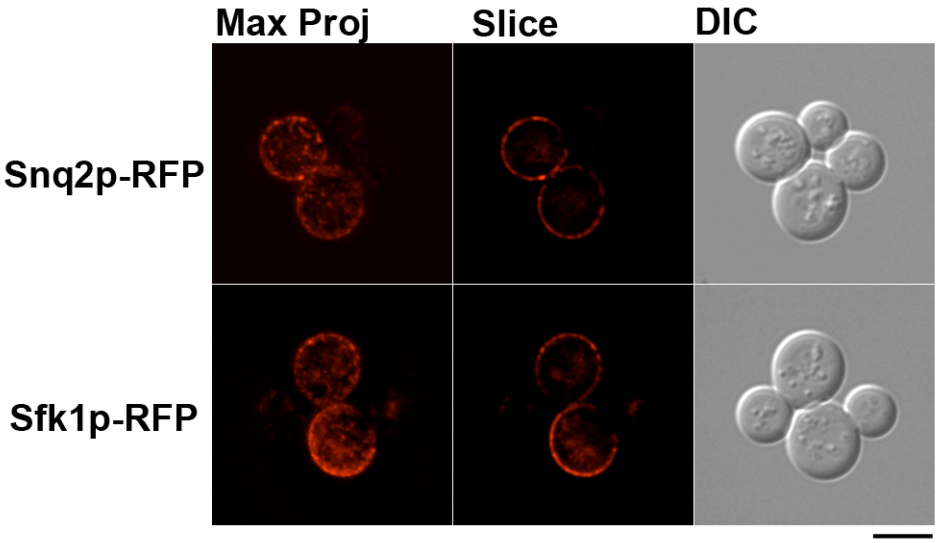


Figure 8.5. RFP plasma membrane markers. RFP plasma membrane markers expressed in wildtype cells. A 2D maximum projection of a 2D maximum projection of a deconvolved 3D z-series and a deconvolved slice from the center of the cell are shown with the corresponding DIC image. Scale bar represents 5μm.

plasma membrane plasma membrane. Sfk1p interacts with the PI 4-kinase Stt4p [10]. In addition to the mother cell plasma membrane, Sfk1p-RFP also appears to be present in the vacuole at times. This localization is consistent with the localization reported in the protein localization database which determined these proteins localize to the cell periphery [2].

Peroxisome marker

Peroxisome target sequence 1 (PTS1)-RFP was created to localize to peroxisomes (Figure 8.6). This localizes to puncta representing peroxisomes, confirmed using deletion strains of peroxisome signal sequence recognition factors *PEX5* and *PEX7* as well as a *PEX17*. Pex5p is required for the import of PTS1-containg proteins into peroxisomes and Pex7p is required for the import of PTS2-containing proteins ([11, 12]. Pex17p is a peroxisome membrane protein required for

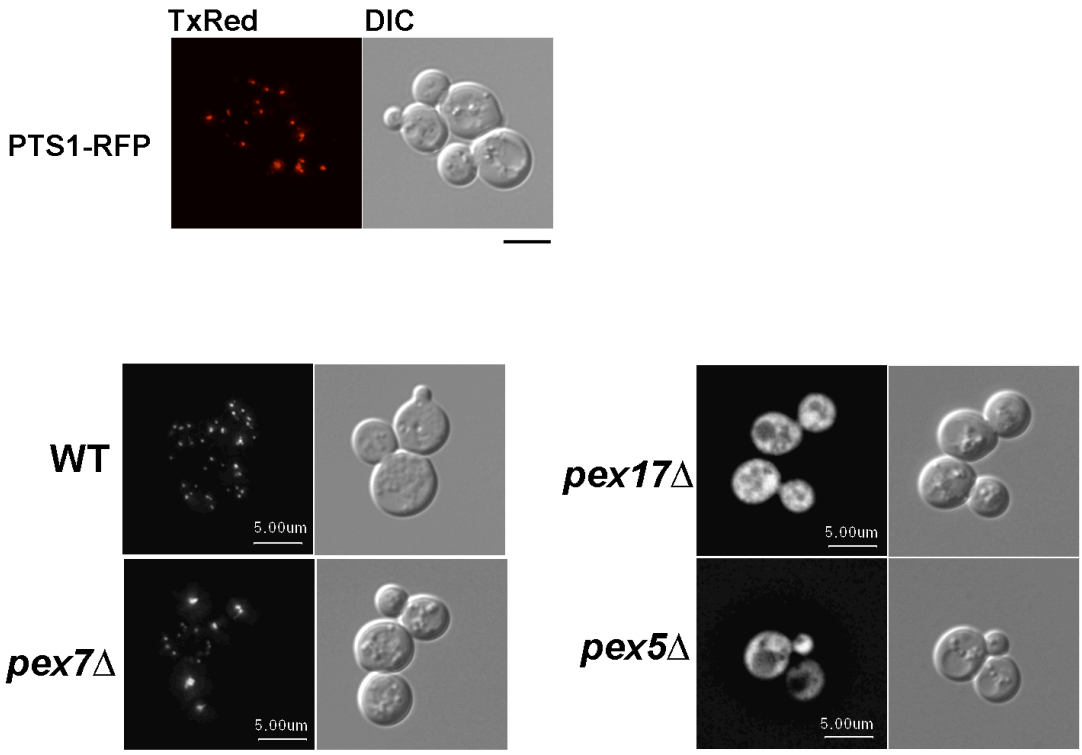


Figure 8.6. RFP peroxisome marker. *(top)* RFP-PTS1 expressed in wildtype cells. A 2D maximum projection of a deconvolved 3D z-series is shown with the corresponding DIC image. (*bottom*) RFP-PTS1 expressed in wildtype, *pex7Δ, pex17Δ,* and *pex5Δ* cells in YNO media. A 2D maximum projection of a 3D z-series is shown with the corresponding DIC image. Scale bar represents 5μm.

the import of PTS1- and PTS2-containing proteins [13]. Under oleic acid induction in wildtype and *pex7Δ* cells, the PTS1-RFP localized to a punctate distribution throughout the cells. However, in *pex17*Δ and *pex5*Δ cells, though, the PTS1-RFP displayed a diffuse localization, demonstrating that PTS1-RFP requires proteins responsible for PTS1 import to peroxisomes for proper localization (Figure 8.6 *bottom*).

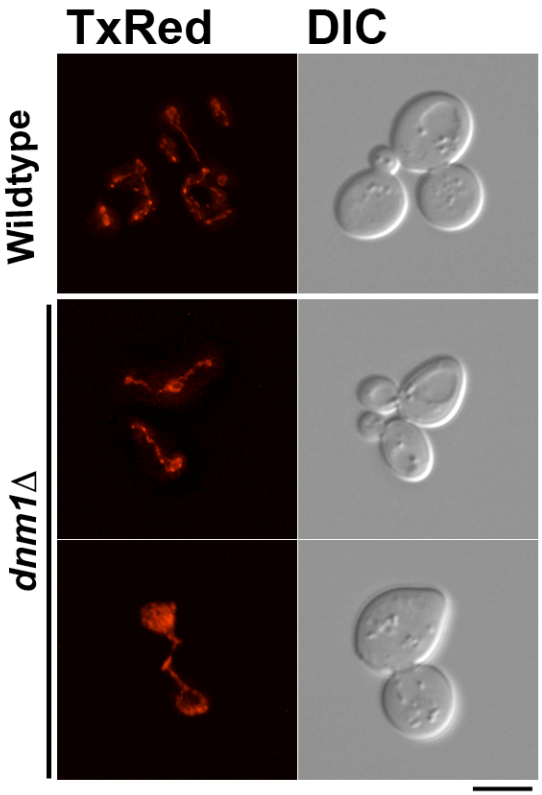


Figure 8.7. RFP mitochondrial marker. RFP-Img1p expressed in wildtype and *dnm1Δ* cells. A 2D maximum projection of a deconvolved 3D z-series and a deconvolved slice are shown with the corresponding DIC image. Scale bar represents 5μm.

Mitochondria marker

Img1p, a mitochondria large ribosome subunit, was tagged with RFP at its C-terminus to create a mitochondria marker (Figure 8.7 *top*) [14]. A mitochondria localization is also reported for an Img1p-GFP fusion protein as determined for the protein localization database [2]. To confirm the mitochondrial localization, Img1p-RFP localization was analyzed in *dnm1Δ* cells. Dnm1p is a dynamin-like GTPase that is involved in mitochondria morphology. In cells lacking Dnm1p function, the mitochondria collapse to one side of the cell, as opposed to being evenly distributed throughout the cell [15, 16]. In addition, a subset of these cells display a mitochondria

network that forms a net-like structure due to defects in mitochondria fission [16]. The same phenotype is observed using Img1p-RFP (Figure 8.7). When compared to wildtype cells, Img1p-RFP is collapsed to one side of the cell in *dnm1Δ* cells. Net structures are also seen in a subset of the *dnm1Δ* cells (Figure 8.7).

Lipid body marker

Four proteins that localize to lipid droplets were tagged with RFP to create lipid droplet markers. Erg6p-RFP, Faa4p-RFP, Fat1p-RFP, and Sna2p-RFP localize to large puncta in the cell (Figure 8.8). An overlay of the fluorescent image with the DIC image reveals that the puncta localize to the light diffracting regions of the cell in the DIC image. This has been reported to be lipid droplets through the use of nile red as a lipid droplet dye [17]. In addition, the lipid droplet markers colocalized with bodipy green, confirming lipid droplet localization (Figure 8.8 *bottom*). The protein localization database for yeast also reports a lipid body localization for these four proteins, although also includes a vacuolar membrane localization for Sna2p-GFP [2]. We fail to see this localization with the Sna2p-RFP generated in this study.

Vacuole markers

The vacuole in yeast can be observed through DIC as a dimpled portion of the cell. Vac14p-RFP, a protein that regulates PtdIns(3,5)P(2) synthesis [18], can be seen as a ring around the vacuole, labeling the limiting vacuolar membrane (Figure 8.9a). This marker, however, does not have a uniform localization but is present more in certain areas of the vacuolar membrane than others. The protein localization database reported that Vac14p-GFP also localizes to the vacuolar membrane [2]. $Cpy_{(1-50)}$-RFP is a truncated version of carboxypeptidase, a vacuolar hydrolase, fused to RFP and can be observed primarily in the vacuolar lumen and to a few puncta in the cell (Figure

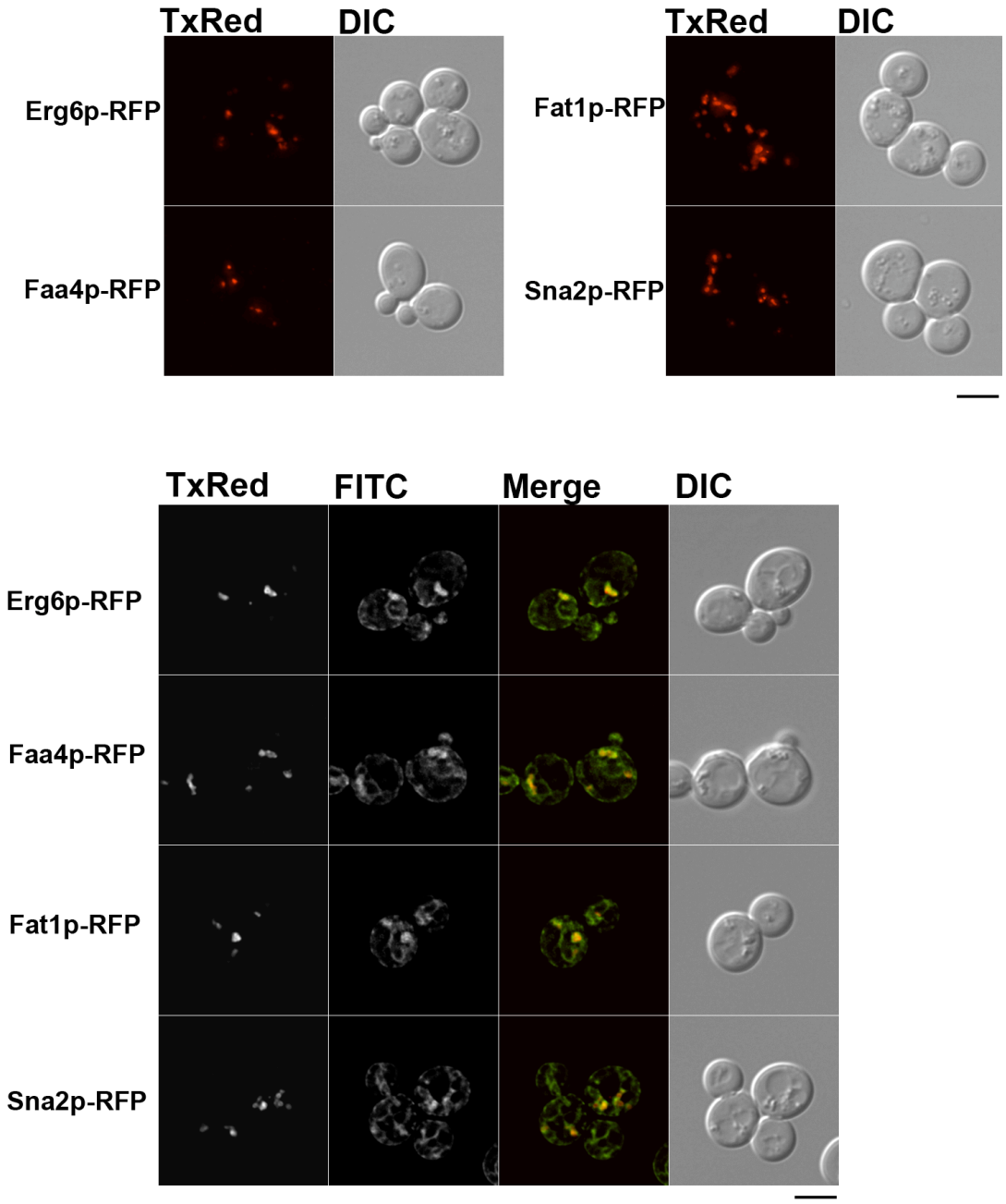
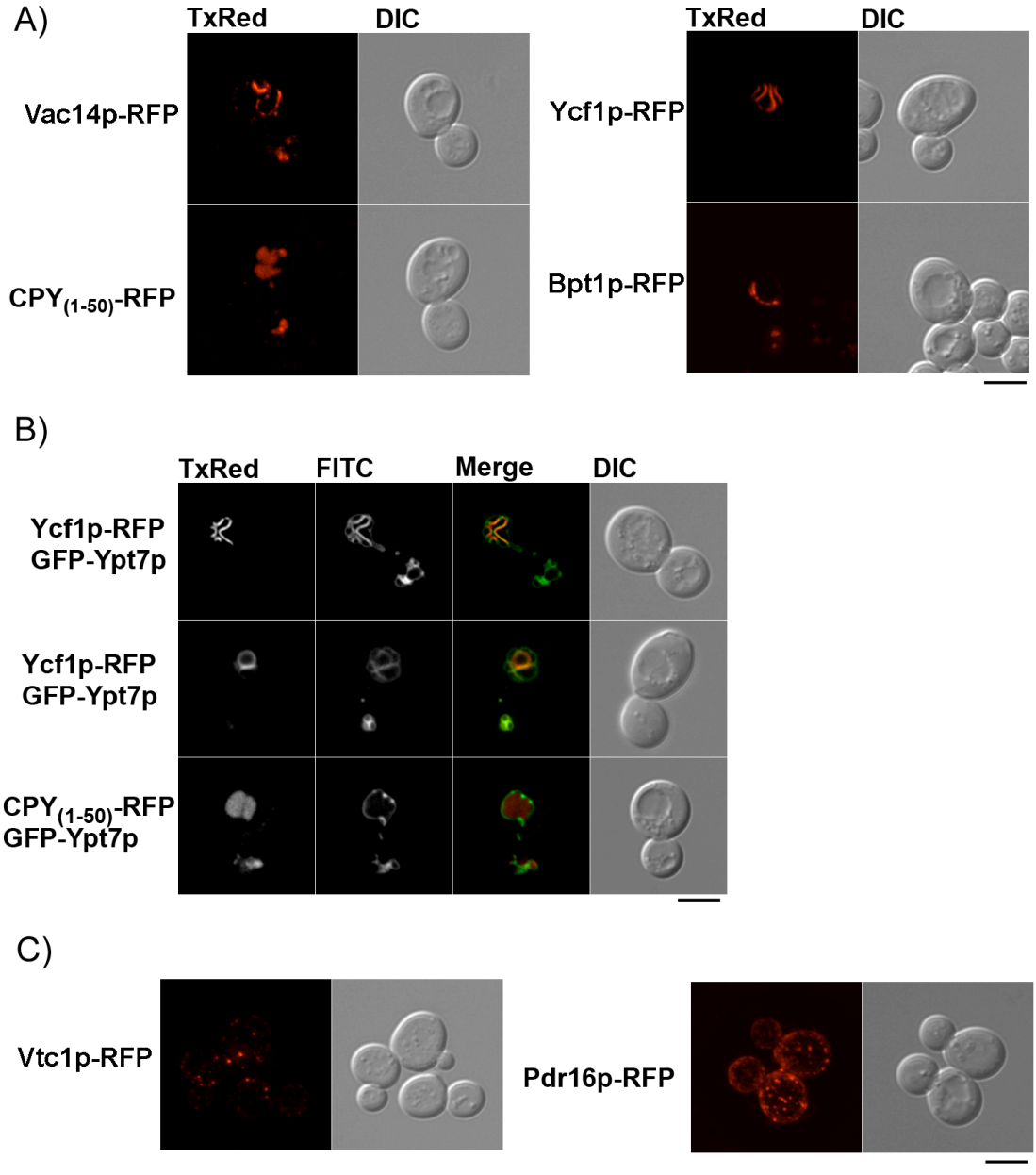


Figure 8.8 RFP lipid body markers. (*top*) RFP lipid body markers expressed in wildtype cells. A 2D maximum projection of a deconvolved 3D z-series is shown with the corresponding DIC image. (*bottom*) RFP lipid body marker colocalization with Bodipy green dye in wildtype cells. A deconvolved fluorescent slice of each channel from the identical z-plane is displayed with the corresponding DIC image. Scale bar represents 5μm.

Figure 8.9. RFP vacuole markers. A) RFP vacuole markers expressed in wildtype cells. A deconvolved slice from the cell center (Vac14p-RFP, CPY-RFP) or a 2D maximum projection of a deconvolved 3D z-series (Ycf1p-RFP) is shown with the corresponding DIC image. B) Colocalization of Ycf1p-RFP and CPY-RFP with GFP-Ypt7p in wildtype cells. A 2D maximum projection of a deconvolved 3D z-series (Ycf1p-RFP, GFP-Ypt7p top example) or a deconvolved slice from the cell center (Ycf1p-RFP, GFP-Ypt7p bottom example and CPY-RFP, GFP-Ypt7p) is displayed with the corresponding DIC image. C) Localization of markers with unidentified localization in wildtype cells. A 2D maximum projection of a deconvolved 3D z-series is shown with the corresponding DIC image. Scale bar represents 5μm.


A)
TxRed
DIC
Vac14p-RFP
CPY(1-50)-RFP
TxRed
DIC
Ycf1p-RFP
Bpt1p-RFP
B)
TxRed
FITC
Merge
DIC
Ycf1p-RFP
GFP-Ypt7p
Ycf1p-RFP
GFP-Ypt7p
CPY(1-50)-RFP
GFP-Ypt7p
C)
Vtc1p-RFP
Pdr16p-RFP

8.9a). Colocalization studies with GFP-Ypt7p, the Rab GTPase that localizes to the vacuolar membrane, confirmed that $Cpy_{(1-50)}$-RFP does localize to the lumen (Figure 8.9b).

Ycf1p-RFP is a fusion protein that localizes inside the vacuole. The localization of this ABC transporter is not in the lumen of the vacuole, however, but appears to localize on internal structures [19]. Overlaying the fluorescent images with the DIC image of the yeast illustrates that this fusion protein localizes to membrane vertices between tethered vacuoles (Figure 8.9a). Colocalization studies with GFP-Ypt7p reveals that Ycf1p-RFP localizes on internal vacuolar membrane structures, which we believe may be the vertices of fusing vacuoles (Figure 8.9b). According to the protein localization database, Ycf1p-GFP is reported to localize to the limiting vacuolar membrane [2]. Our colocalization analysis found that Ycf1p-RFP is concentrated solely in vacuolar membrane vertices and therefore suggests that the RFP moiety may interfere with protein localization.

Vtcp1p is a protein that has been reported to localize to the vacuole when tagged with GFP [20]. The yeast localization database found Vtc1p-GFP to localize to the ER [2]. A Vtcp1p-RFP fusion protein, however, localizes to puncta in cells (Figure 8.9c). The puncta may represent endosomes en route to the vacuole or another punctate subcellular structure. Therefore, this suggests that tagging Vtc1p with RFP alters its localization as reported by GFP fusion proteins.

Pdr16p-RFP, a protein involved in phosphinositide transport, was also generated. Pdr16p-RFP localizes to puncta near the edge of the yeast cell (Figure 8.9c), however, this marker does not colocalize with GFP-Vps21p or GFP-Ypt53p (not shown). GFP-fusions of Pdr16p have been reported to localize to lipid particles and microsomes [2, 21], however, we have been unable to characterize the Pdr16p-RFP localization.

Endosome markers

Endosomal RFP-fusion proteins were generated. Tvp23p-RFP and Ypt35p-RFP, two proteins of unknown function, and the sorting nexin Snx41p-RFP [22], were created that localize to puncta in the cell (Figure 8.10a). Colocalization studies were performed using GFP-Vps21p, the Rab GTPase that localizes to endosomes to determine if the puncta are indeed endosomes. Snx41p-RFP, Ypt35p-RFP and Tvp23p-RFP were all found to colocalize with GFP-Vps21p (Figure 8.10b). In each case, not all GFP-Vps21p colocalized with the RFP endosomal marker, suggesting that these endosomal markers represent a subset of the endosomal pathway. In addition, a subset of Tvp23p-RFP showed colocalized with GFP-Vps21p per cell, suggesting that this marker represents a small class of endosomes and an additional compartment. Consistent with the colocalization analysis, the protein localization database found that GFP tagged versions of Tvp23p localizes to endosomes and the late Golgi apparatus [2]. In addition, Snx41p-GFP was reported to localize to endosomes and lipid particles [2].

Discussion

The markers described here could be used in the future for many applications, such as colocalization studies with proteins of unknown localization. Another powerful application is to use these markers to monitor organelle changes over time caused by certain mutations. This could give insights into protein function and its impact on organelle architecture. The localization of the majority of the RFP markers described here is consistent with the GFP protein localization database [2].

Ycf1p-RFP displays an intriguing localization. In a three-dimensional context, the tethering of multiple vacuoles together creates a shared membrane domain

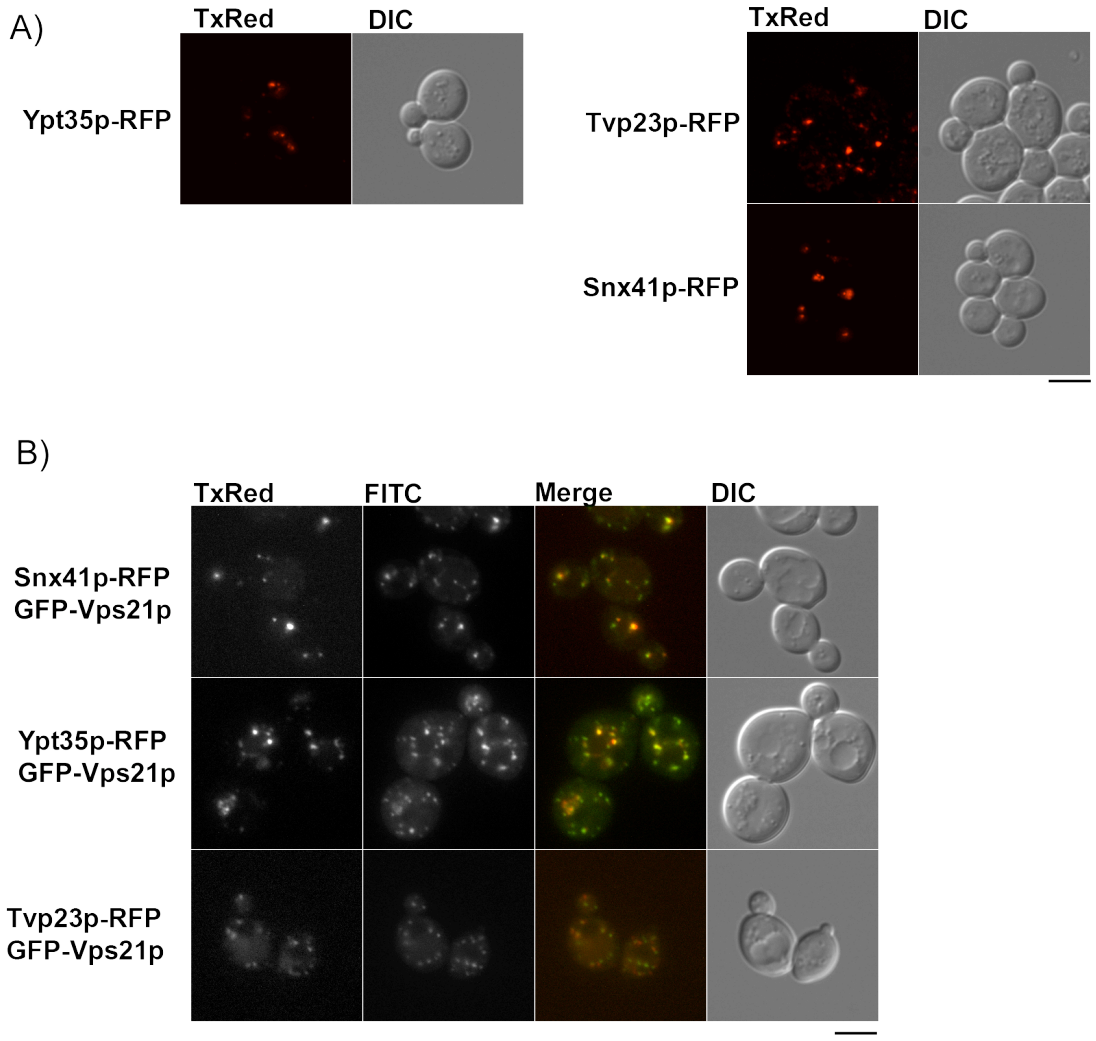


Figure 8.10. RFP endosome markers. A) RFP endosome markers in wildtype cells. A 2D maximum projection of a deconvolved 3D z-series is shown with the corresponding DIC image. B) RFP endosome marker colocalization with GFP-Vps21p in wildtype cells. Images from each channel in a single plane are shown with the corresponding DIC image. Scale bar represents 5μm.

between two tethered vacuoles forms the vacuole [23]. Figure 8.9 illustrates this feature of the vacuole where GFP-Ypt7p is used as a vacuolar membrane marker. Colocalization analysis with GFP-Ypt7p reveals that Ycf1p-RFP localizes to the vacuolar vertex membranes. Wickner and colleagues found that Vam7p-GFP also is enriched in vacuolar vertices and using an *in vitro* tethering assay found that Gdip1p,

Vam3p and Gyp1p are required for vacuolar tethering [23]. Ycf1p-RFP could also be used as a marker to identify proteins where loss of function results in depleted vacuolar tethering events or excessive vacuolar tethering events.

This study describes the subcellular localization of a new set of RFP fusion proteins generated in shuttle plasmids that can be used by the *S.cerevisae* community for studying a number of organelles. Initial characterization of each marker is presented to verify the intracellular compartment of localization. The markers are versatile and can be used for live cell microscopy or with fixed cells, as they are genetically encoded fluorescent reporters.

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Chapter 9. A plasma membrane subdomain that contains the ABC transporter Snq2p

Abstract

Cellular asymmetry is an evolutionarily conserved feature of eukaryotic cells. Cells distribute their organelles to allow for organelle inheritance during cell division. Additionally, organelles are structured into subdomains to create specialized functional zones. We have investigated the localization of the ABC transporter, Snq2p, a transmembrane protein that is asymmetrically concentrated in the mother cell plasma membrane for a large portion of the cell cycle. Our data suggests Snq2p travels through the secretory pathway but is concentrated away from sites of exocytosis, where newly synthesized lipids and membrane proteins are deposited, but requires endocytosis to maintain an asymmetric distribution. Many proteins have been identified to localize to sites of exocytosis. Our study demonstrates Snq2p is concentrated away from sites of exocytosis and spatially regulated into a subdomain of the plasma membrane.

Introduction

Cells arrange their contents in an asymmetric pattern both at the cellular level, where organelles are asymmetrically distributed within the cell, and at the subcellular level, creating specialized functional zones within organelles. Polarization is an evolutionarily conserved feature of eukaryotic cells and can be studied in cell types including neurons, polarized epithelia, and *Saccharomyces cerevisiae* [1-4]. *S. cerevisiae* is a model system to study cellular asymmetry because of their ability to polarize cell growth and segregate organelles in the daughter cell prior to cell division [2].

Current models of polarized growth indicate that signalling cues direct polarization and nucleation of actin cables to direct secretory vesicle transport towards the daughter cell plasma membrane. Secretory vesicle fusion with the plasma membrane provides the membrane required to increase surface area to allow for growth. As a consequence, newly synthesized lipids and integral membrane proteins are deposited in the growing plasma membrane at the bud [5].

Reports have demonstrated that the plasma membrane in *S.cerevisiae*, although a continuous lipid bilayer, is arranged in subdomains including lipid rafts, eisosomes, and the membrane compartment occupied by Pma1p (MCP) [6-11]. The organization of these microenvironments presumably allow for specialized functional regions of the cell surface, for example, localizing exocytic SNAREs at the sites of exocytosis [12, 13]. The localization of Fus1p, a protein involved in cell fusion during mating, and other membrane proteins that localize to the daughter cell plasma membrane can be achieved by transportation on secretory vesicles and deposition in the daughter cell plasma membrane [14, 15]. Therefore, exocytosis is involved in establishing the polarized localization of these proteins. Endocytosis and a septin diffusion barrier have been two mechanisms shown to be required to maintain the polarized localization of certain proteins [7, 8, 16, 17].

Here we describe the localization of the well-characterized ABC transporter Snq2p [18-20]. Snq2p is a transmembrane protein that at steady state localizes to the mother cell plasma membrane for a large portion of the cell cycle and is largely excluded from sites of exocytosis. We find that this protein travels through the secretory pathway and can be trapped in secretory vesicles, suggesting that it can get to the bud, however at steady-state is largely concentrated away from this area. We hypothesize that Snq2p may be sequestered once it is deposited in the plasma

Table 9.1. Yeast strains used in this study

Strain Number	Genotype	Source
RCY239	*MAT***a** *ura3-52 leu2-3,112*	Collins Laboratory
RCY252	*MAT***a** *ura3-52 leu2-3,112 sec6-4*	Novick Laboratory
RCY3150	*MAT***a** *ura3-52 leu2-3,112 sec18-1*	Collins Laboratory
SHY625	*MAT***a** *ura3 leu2 his3 lys2 trp1 sec14-3*	Henry Laboratory
RCY4053*	*MAT***a** *ura3 leu2 his3*	Collins Laboratory
M-239	*MAT***a** *ura3 leu2 his3 trp1 cdc12-6*	Longtine Laboratory
RH144-3D	*MAT**a** ura3 his4 leu2 bar1-1*	Reizman Laboratory
RH268-1C	*MAT**a** ura3 his4 leu2 bar1-1 end4^{ts}*	Reizman Laboratory

* isogenic control for *cdc12-6* generated through isolating temperature resistant spores from crossing Longtine laboratory strains M-12(*MATα ura3 leu2 cdc3-3*) x M-195(*MAT***a** *ura3 leu2 his3 cdc10-1*).

membrane at the bud tip, possibly through endocytosis, as it requires endocytosis to maintain its restricted localization to the mother cell plasma membrane.

Materials and Methods

Yeast strains, plasmids and media

Yeast strains used in this study are listed in Table 9.1. Plasmids used in this study are listed in Table 9.2. YPD (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), synthetic complete dropout (0.1% yeast nitrogen base, 2% D-glucose, synthetic complete dropout mixture), SD (0.1% yeast nitrogen base, 2% D-glucose, plus required nutrients), synthetic complete sucrose dropout (0.1% yeast nitrogen base, 2% sucrose, synthetic complete dropout mixture), and Sgal (0.1% yeast nitrogen base, 2% galactose plus required nutrients) media was used.

Microscopy

Live cells were analyzed with a Nikon Eclipse E600 microscope, 100X (1.4 NA) lens, 1X optavar (0.08 μm/pixel), and imaged using a Sensicam EM High

Table 9.2. Plasmids used in this study.

pRC2098	GFP-Sec4p pRS316	This study
pRC2147	Sec3p-GFP pRS316	[22]
pRC3178A	GFP2-CBP80NLS pRS316	This study
pRC2780A	Snq2p-GFP pRS315	This study
pRC2792A	Snq2p-RFP pRS315	This study
pRC2715	RFP-Gal4BP pRS316	This study
pRC1243	GFP-Ypt7p pRS316	[23]
pRC3334C	Fus1p-GFP P_{GALS} pRS315	This study
pRS3343	Snq2p-GFP P_{GALS} pRS315	This study
pRC3360A	Snq2p-RFP P_{GALS} pRS316	This study
pRC3333A	Mid2p-GFP pRS315	This study
pRC2125	RFP-Sec2p pRS315	This study
pRC2250	KDEL-RFP pRS315	This study

Performance camera (The Cook Corporation). Differential interference contrast (DIC) images were taken at one plane. Fluorescent images were collected using deconvolution microscopy with a 0.2 μm z step size. RFP-tagged proteins were analyzed with a TxRed filter (excited at 540-580 nm and emission at 600-660 nm). GFP-tagged proteins were analyzed with a FITC filter (excitation at 465-495 nm and emission at 515-555). Images were captured with IP Lab 3.6.5 software and blind deconvolution with 30 iterations was done using Autodeblur and Autovisualize 9.1 software.

For microscopy using temperature sensitive mutants, transformants were grown overnight at room temperature in minimal media. Cells were temperature shifted to 37ºC for the indicated amount of time.

Snq2p P_{GALS}–containing strains were grown to stationary phase (grown for approximately 2 days) in synthetic complete media with 2% sucrose as the sole carbon source. This was used to inoculate a fresh sucrose culture to log phase grown overnight. The cells were switched to grow in 2% galactose for the indicated amount of time. Snq2p-GFP P_{GALS} localization in wildtype, *sec18-1, sec14-1,* and *sec6-4* cells

was done by initiating expression for two hours at room temperature with 2% galactose. Cells were then shifted to 37^{o}C for three hours (secretion block) or remained at room temperature (room temperature control).

G_0 isolation was done as described in [21]. Cultures were split and mating factor (a factor) was added to 2μg/ml to one of the samples upon adding glucose (carbon source).

FRAP analysis of Snq2p-GFP and Mid2p-GFP was done with a Leica Upright DMRE-7 Confocal with a 40x oil objective (1.25 NA). Indicated area (arrows) was bleached with maximum argon laser power (488nm) with 5 pulses at 1.65 seconds each. Recovery after photobleaching was monitored over time.

Results and Discussion

Snq2p displays a cell cycle dependent localization and is concentrated away from sites of exocytosis

Our laboratory generated a set of RFP-tagged subcellular compartment markers (previous chapter) to aid in colocalization studies. The zenobiotic transporter *SNQ2* was tagged at its C-terminus and at steady state localized to the mother cell plasma membrane but appeared to be concentrated away from the daughter cell plasma membrane. This observation was intriguing as new lipid and plasma membrane proteins are deposited in the daughter cell plasma membrane and therefore we wished to investigate how Snq2p was able to maintain its localization.

When observed at different stages through the cell cycle, Snq2p-RFP is concentrated towards the mother cell plasma membrane for a large portion of the cell cycle, where cell cycle progression was monitored by bud size and nuclear positioning using a GFP tagged nucleus marker (Figure 9.1a). Generally, once the nucleus

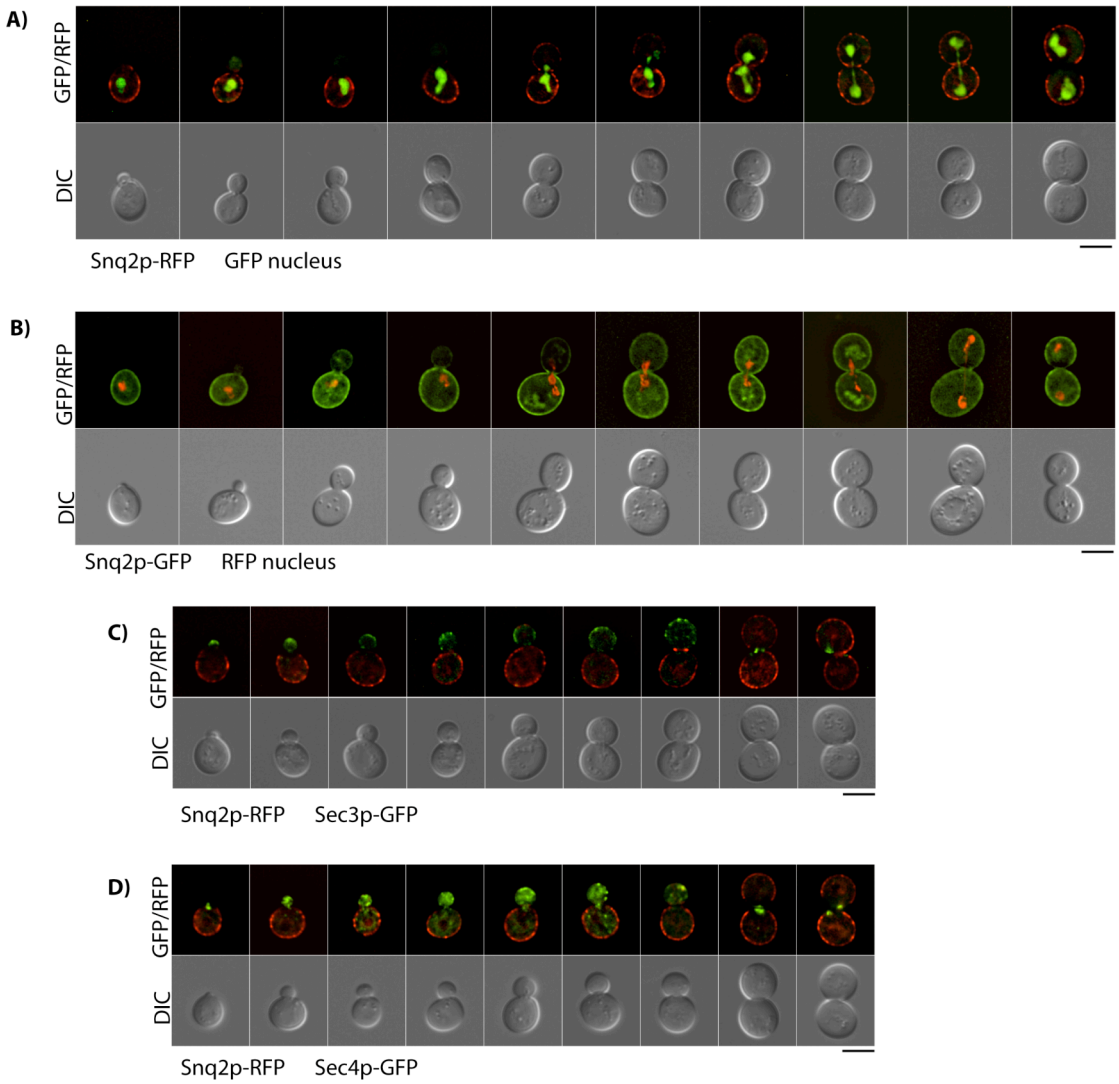


Figure 9.1. Snq2p steady-state localization. A) Snq2p-RFP steady state localization with repsect to progression through the cell cycle (monitored using a GFP nuclear marker). B) Snq2p-GFP steady state localization with respect to progression of the cell cycle (monitored using an RFP nuclear marker). C) Snq2p-RFP localization with respect to the polarity marker Sec3p-GFP and D) a marker for sites of exocytosis, GFP-Sec4p. A deconvolved slice from a deconvolved 3D z-series was selected from the center of the FITC and TxRed stacks and represented with the corresponding DIC image. Size bar corresponds to 5μm.

migrates into the daughter cell Snq2p-RFP localizes to the daughter cell plasma membrane.

An explanation for the localization could be that the RFP tag on the protein plays a role in the asymmetric localization. The version of RFP used to create the fusion protein requires a tetrameric oligomerization of the RFP moiety to produce a fluorescent signal. It is possible that the RFP requires a maturation period similar to the amount of time it takes the cells to undergo almost a full cell cycle to produce a signal. An RFP-Snc2p or RFP-Sec2p fusion protein has a cell cycle dependent localization and can be found in the daughter cell and produce signal at the early stages of the cell cycle, suggesting this is probably not the case (Chapter 8). Further more, the asymmetric distribution of Snq2p is independent of RFP as Snq2p-GFP is also concentrated towards the mother cell plasma membrane, displaying a similar cell cycle-dependent localization (Figure 9.1b). It is worth noting that Snq2p-GFP displays an asymmetric localization and is concentrated in the mother cell plasma membrane but there appears to be weak fluorescent signal in the daughter cell plasma membrane, suggesting that it may be present at very small amounts in this region. Colocalization studies with the polarity marker Sec3p-GFP and the exocytic marker GFP-Sec4p revealed that Snq2p-RFP is concentrated away from sites of exocytosis (Figure 9.1c,d) [24, 25]. When exocytosis is directed towards the daughter cell periphery at early stages in the cell cycle, Snq2p is concentrated away from this region. It appears that when exocytosis is redirected towards the mother-daughter cell interface, Snq2p is localized in the daughter cell periphery.

It is intriguing that an integral membrane protein is largely absent from sites of exocytosis and displays this cell cycle dependent localization. We wondered if this could be explained through a cell cycle dependent expression pattern. Constitutive expression with the galactose-inducible promoter results in an asymmetric localization

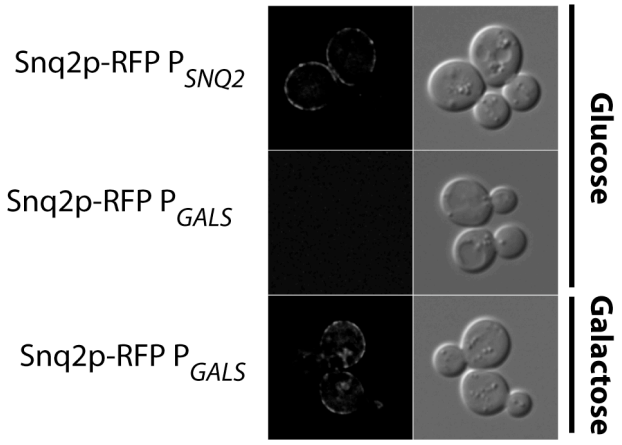


Figure 9.2. Snq2p localization is independent of transcriptional control. Snq2p-RFP containing cells grown in either minimal media containing glucose (P_{SNQ2}) or galactose (P_{GALS}). A deconvolved slice from a deconvolved 3D z-series is shown with its corresponding DIC image. Size bar corresponds to 5μm.

similar to expression under its endogenous promoter indicating that localization is independent of transcriptional regulation (Figure 9.2).

Snq2p is a transmembrane protein that can be found in compartments throughout the secretory pathway.

Topology prediction using the *SNQ2* primary sequence and the topology algorithm TopPred reveals that Snq2p is a transmembrane protein with 12 or 13 predicted transmembrane regions [26]. Twelve of the transmembrane domains are predicted certain domains. If the protein adopts this topology both the N- and C-termini are predicted to be oriented towards the cytosol (Figure 9.3a). If the one putative transmembrane segment is inserted into the predicted topology then the N- and C-terminus are oriented towards opposite environments. Proteins that traverse the secretory pathway are often glycosylated and undergo processing of the glycosylation

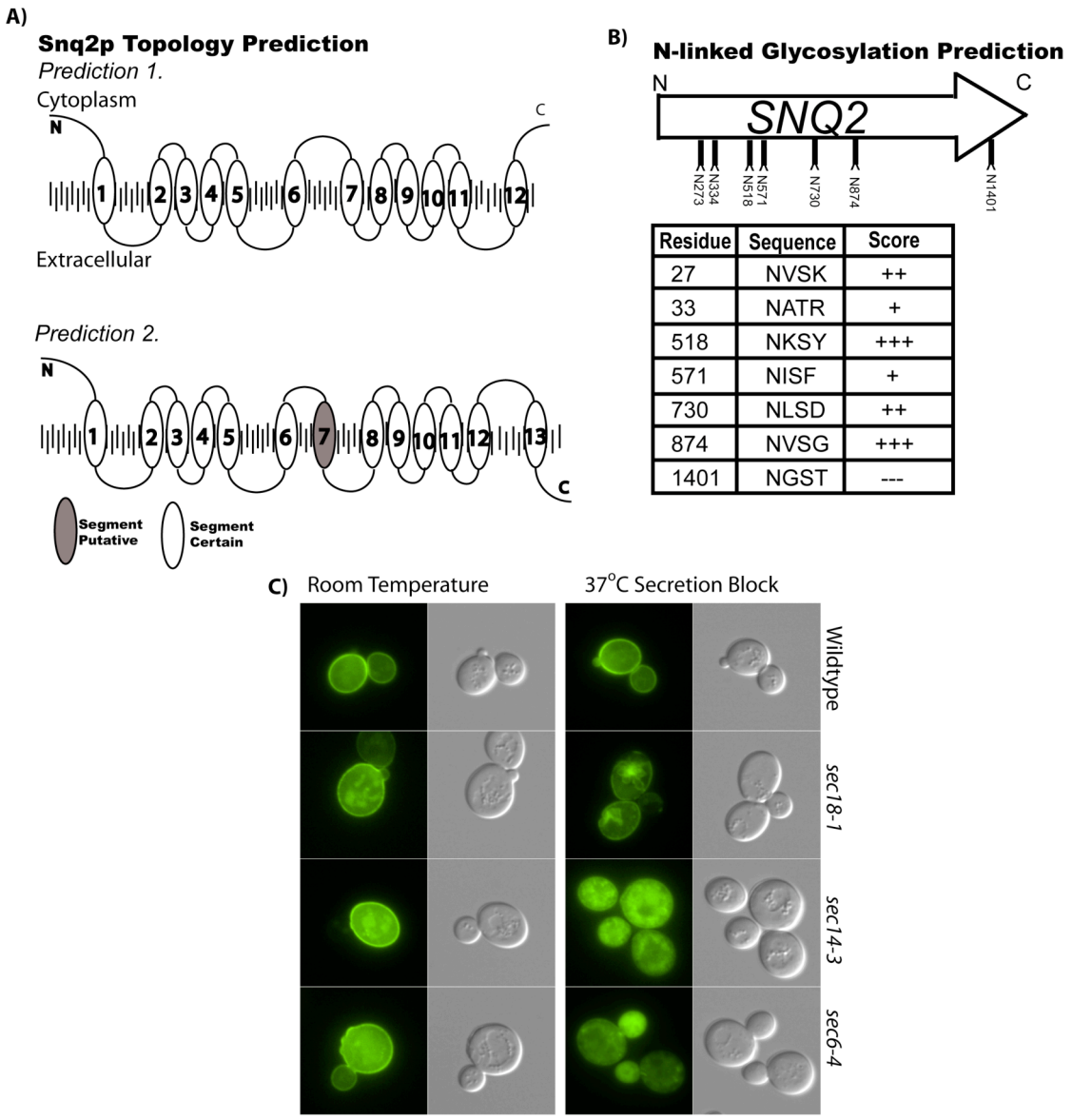


Figure 9.3. Snq2p-GFP travels through the secretory pathway. A) Snq2p topology prediction from TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html). B) N-linked glycosylation prediction of Snq2p residues from NetNGlyc 1.0 server. C) Snq2p-GFP P_{GALS} localization in wildtype, *sec18-1, sec14-1,* and *sec6-4* mutant cells. Expression was initiated for two hours at room temperature with 2% galactose. Cells were then shifted to 37°C for three hours (secretion block) or remained at room temperature (room temperature control).

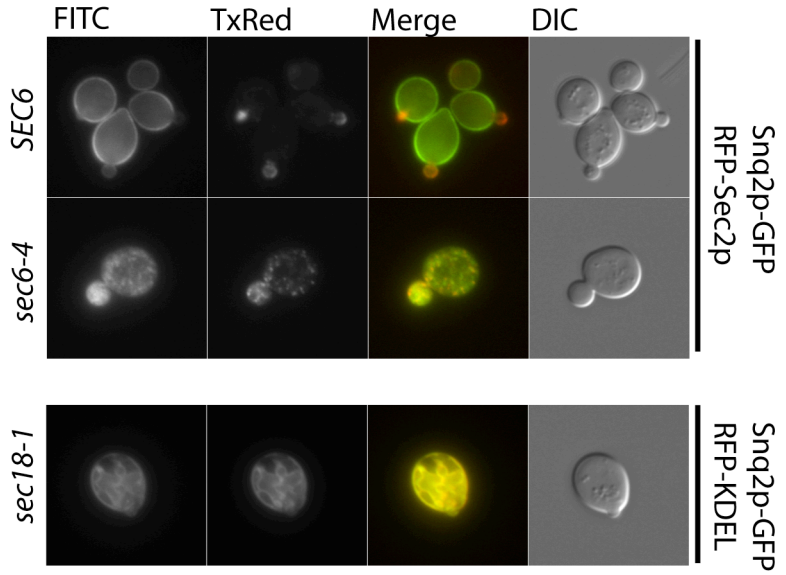


Figure 9.4. Colocalization of Snq2p-GFP with intracellular markers in *sec* mutants. Cells were grown overnight in minimal media containing sucrose. The galactose inducable promoter controlled Snq2p-GFP expression. Expression was initiated for two hours at room temperature by changing to minimal media containing galactose. Cells were then shifted to 37^{o}C for three hours. Single images were taken in each filter indicated.

modification in the Golgi apparatus. We were interested in determining if Snq2p travels through the secretory pathway [5].

NetNGlyc 1.0 predicts Snq2p to be N-linked glycosylated at multiple sites with high confidence (Figure 9.3b) [27]. *In vivo* Snq2p can be trapped in compartments throughout the secretory pathway when expression is controlled with the galactose inducible promoter and secretion is blocked using temperature sensitive secretion mutants. In *sec18-1* mutants, which causes a general secretion block at restrictive temperature, Snq2p-GFP accumulates in the endoplasmic reticulum at restrictive temperature (Figure 9.3c) [28]. Presumably, Snq2p is inserted in the ER bilayer and because all trafficking stages are blocked, is unable to exit the ER. ER localization was confirmed through colocalization studies in *sec18-1* with KDEL-RFP, a luminal

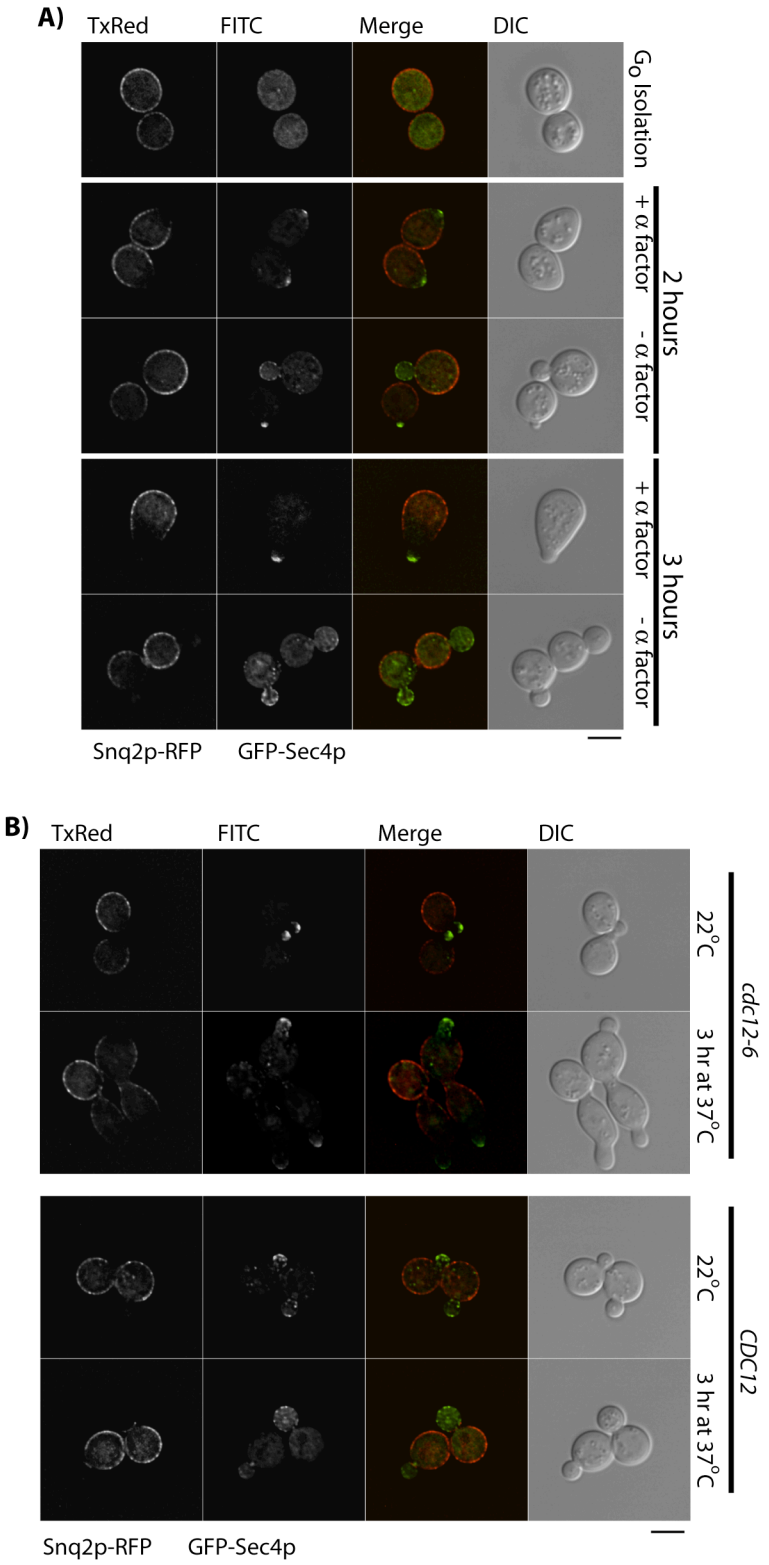
ER marker (Figure 9.4). Traffic exiting the Golgi is blocked in *sec14-3* mutants [29]. Upon secretion block in *sec14-3*, Snq2p-GFP accumulates in a punctate pattern resembling an accumulated Golgi apparatus, suggesting Snq2p-GFP enters the Golgi at some point throughout its journey to its resident domain (Figure 9.3c). Delivery of post-Golgi vesicles to the plasma membrane is blocked in *sec6-4* mutants at restrictive temperatures [30]. Snq2p-GFP accumulates in a compartment that accumulates in the bud tip in *sec6-4* cells at restrictive temperature, presumably caused by the defect in delivery to the plasma membrane (Figure 9.3c). These vesicles were found to be secretory vesicles through colocalization with RFP-Sec2p, a Rab guanine nucleotide exchange factor found on secretory vesicles (Figure 9.4) [31].

Snq2p does not require the septin as a diffusion barrier

The septin between the mother and daughter cell could create a diffusion barrier to restrict free diffusion of Snq2p to the daughter cell plasma membrane, as is the case for Ist2p [8]. Two approaches were used to assess if Snq2p requires the physical barrier to maintain localization by analyzing localization in yeast extending their mating projections and in cells with temperature sensitive septins.

Yeast can be two mating types, either **a** or α. When mating type **a** cells are subjected to α mating factor they undergo polarized growth to extend their mating projection towards the mating factor. A culture was isolated that was enriched in G_0 cells [21]. The culture was split and cells were grown with or without α mating factor. GFP-Sec4p was used as a marker for sites of polarized growth [24]. Similar to vegetative growth, Snq2p-RFP is concentrated away from sites of polarized growth in cells extending their mating projections and remains at the posterior of the cell (Figure 9.4a).

Figure 9.5. Snq2p-RFP does not require the septin as a physical barrier to maintain localization. A) A culture with a high enrichment in G_0 cells (described in materials and methods) was split and grown with (2μg/ml) and without α factor. Snq2p-RFP and GFP-Sec4p localization is shown using a deconvolved slice from a deconvolved 3D z-series alongside its corresponding DIC image. B) Snq2p-RFP and GFP-Sec4p localization in *CDC12* and *cdc12-6* cells after growth at room temperature or 37^{o}C for three hours. A deconvolved slice from a deconvolved 3D z-series alongside its corresponding DIC image is shown. Size bar corresponds to 5μm.


A)
TxRed
FITC
Merge
DIC
G_0 Isolation
+ α factor
- α factor
2 hours
+ α factor
- α factor
3 hours
Snq2p-RFP
GFP-Sec4p
B)
TxRed
FITC
Merge
DIC
22°C
3 hr at 37°C
cdc12-6
22°C
3 hr at 37°C
CDC12
Snq2p-RFP
GFP-Sec4p

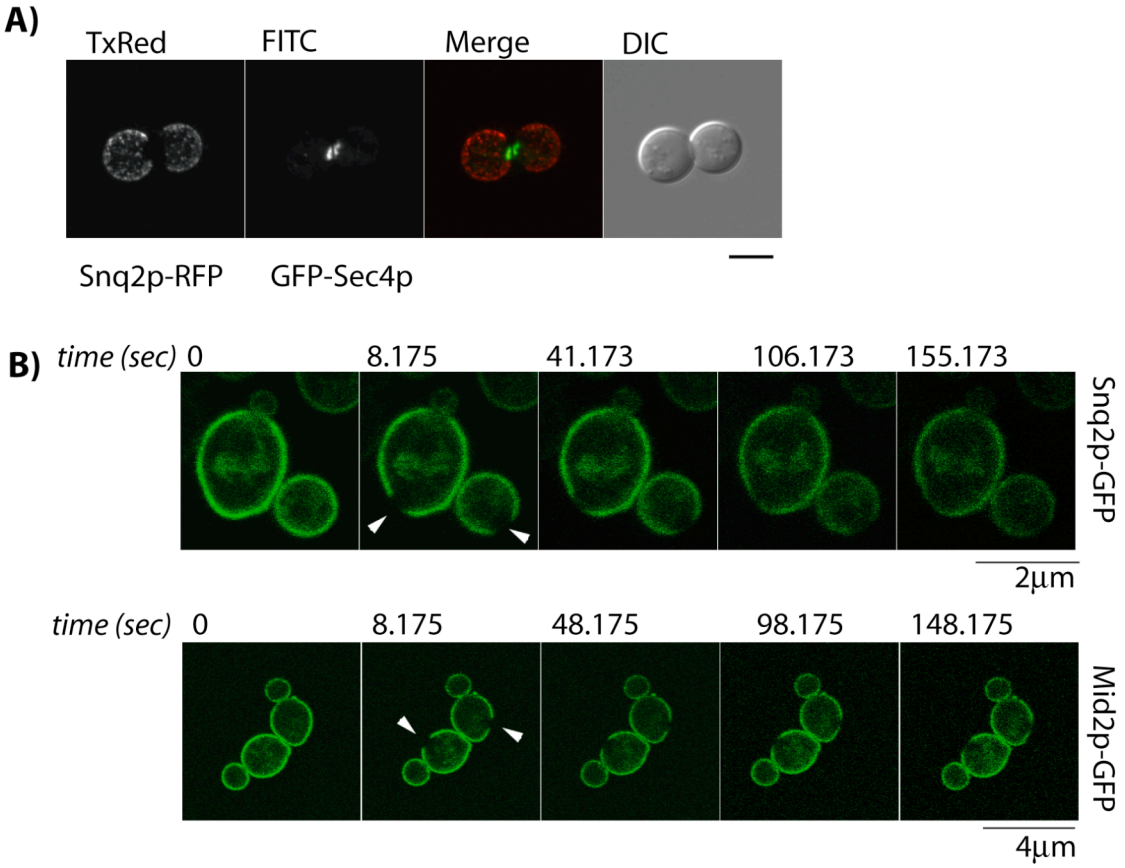


Figure 9.6. Snq2p appears to be mobile in the plasma membrane. A) A deconvolved maximum projection from a 3D z-series of Snq2p-RFP and GFP-Sec4p localization in a wildtype cell at cytokinesis. B) FRAP analysis of Mid2p-GFP and Snq2p-GFP mobility in the plasma membrane. An area on the plasma membrane was bleached (white arrows) and fluorescent signal was monitored over time. Note the size bars – different zooms were used for the two images of cells shown.

Septins are depleted from cells when shifted to restrictive temperature in the mutant *cdc12-6 [32]*. Cells approaching cytokinesis without septins fail to separate and therefore form tandem buds. Snq2p localization was monitored in cells depleted septins using this mutant. In these cells Snq2p-RFP is concentrated away from sites of exocytosis (GFP-Sec4p), even when the mutant cells exocytose at more than one bud site, as seen in Figure 9.4b in *cdc12-6* cells after 3 hours of growth at 37^{o}C.

An additional observation supporting that Snq2p localization is independent of the septin as a physical barrier is seen when looking at a 3D reconstruction of a cell undergoing cytokinesis. Secretion is directed towards the neck between the two dividing cells at this point in the cell cycle. Snq2p-RFP is excluded from the neck region of these cells where GFP-Sec4p is used as a marker for sites of exocytosis (Figure 9.5a).

Snq2p appears to be laterally mobile in the plasma membrane using FRAP analysis (Figure 9.5b). Snq2p-GFP has similar recovery characteristics to another plasma membrane protein, Mid2p-GFP (Figure 9.5b), suggesting that the exclusion of Snq2p from sites of exocytosis is not due to an inability of Snq2p to diffuse in the plasma membrane. This data suggests an active process is required to maintain Snq2p steady state distribution.

Snq2p requires active endocytosis to maintain localization

We reasoned there may be a process that could clear Snq2p from sites of exocytosis. Endocytosis is required to maintain an asymmetric plasma membrane in various cell types, including maintaining a polarized localization of Snc1p [7]. Endocytosis is blocked in an *end4*^{ts} mutant at restrictive temperature [33]. When Snq2p-RFP localization was monitored in endocytosis deficient cells, Snq2p-RFP localizes to both the mother cell and daughter cell plasma membrane after endocytosis is blocked for 60 minutes (Figure 9.6a). Prior to shifting the restrictive temperature, Snq2p-GFP is completely absent in the daughter cell. After growth at restrictive temperature for 30 minutes, Snq2p-RFP appears to begin to accumulate in at the daughter cell periphery and by 60 minutes, Snq2p-RFP accumulates even more in the daughter cell periphery. The Snq2p concentration gradient is no longer apparent as it

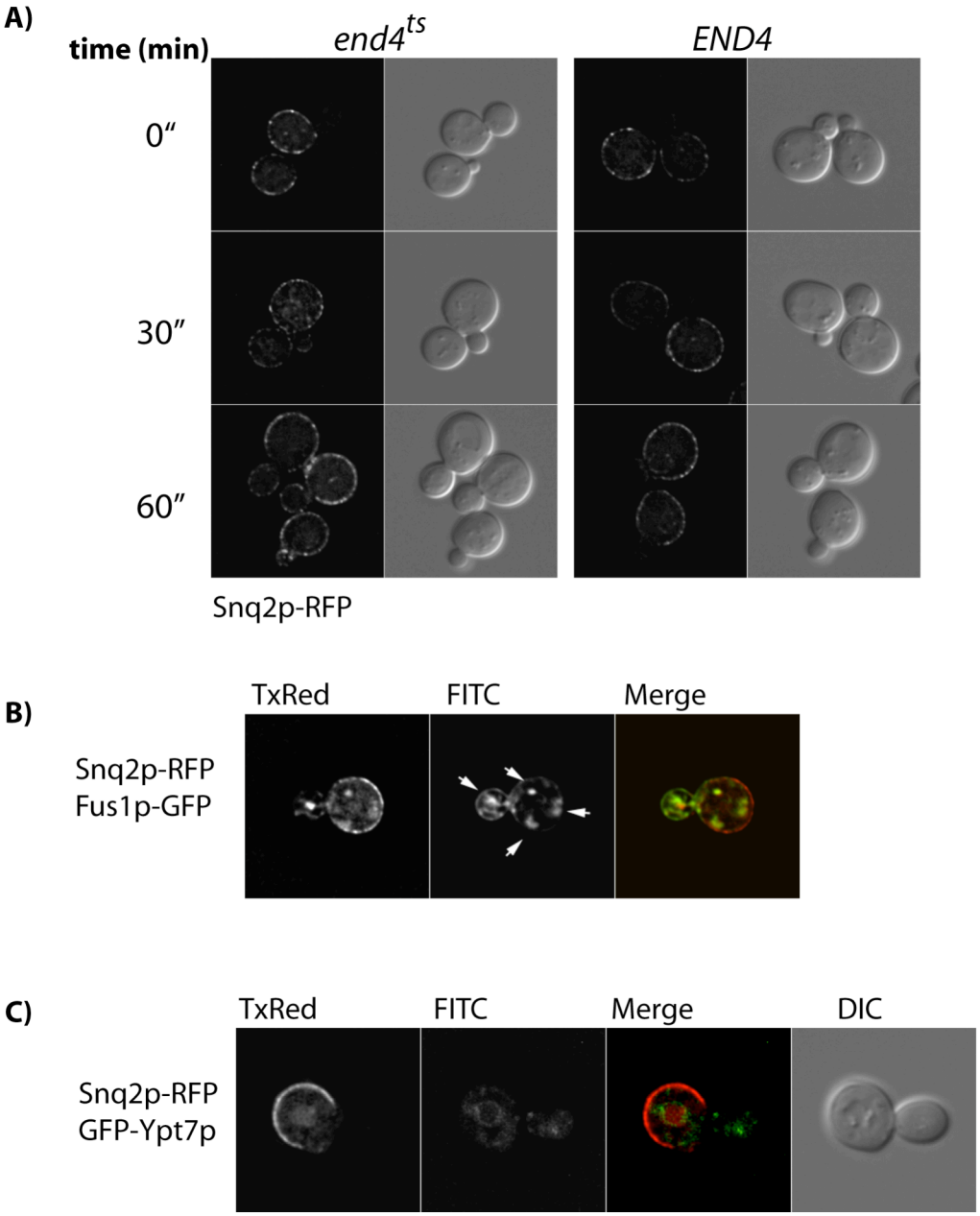


Figure 9.7. Snq2p requires continued endocytosis for asymmetric localization. A) Snq2p localization in *END4* and *end4*^{ts} cells after growth at 37°C. B) Snq2p-RFP P_{GALS} colocalization with Fus1p-GFP P_{GALS} and C) Snq2p-RFP P_{GALS} colocalization with GFP-Ypt7p. Cells were grown overnight (~16 hours) in minimal media containing 2% galactose. A deconvolved slice was selected from the center of the cell. Size bar corresponds to 5μm.

appears to be present at similar levels in both membranes compared to a wildtype cell where it is only localized to the mother cell plasma membrane.

The exact mechanism for how Snq2p is able to achieve its localization is unclear. Data collected to date indicates that Snq2p is an integral membrane protein that travels through the secretory pathway but is concentrated away from sites of exocytosis. However, if endocytosis is blocked Snq2p becomes unpolarized, suggesting that once it is deposited on the daughter cell plasma membrane it may be sequestered and immediately excluded from this domain of the plasma membrane. If this is the case, it is unclear what happens to the protein. When Snq2p-RFP is overexpressed using the galactose-inducable promoter or when GFP tagged under its endogenous promoter some signal can be found in the vacole.

Fus1p-GFP is a transmembrane protein that travels through the secretory pathway that localizes to the daughter cell plasma membrane and the vacuole [14, 15]. Colocalization studies revealed that they each localize to different domains of the plasma membrane, however, colocalize in regions in the cell interior (Figure 9.6b). The regions of colocalization inside the cell appear be the vacuole lumen, as observed through colocalization with the Rab GTPase, GFP-Ypt7p, which regulates vacuolar fusion that localizes to the limiting vacuole membrane (Figure 9.6c). It remains unclear if Snq2p gets degraded via endocytosis and vacuolar proteases as Snq2p-RFP, when expressed under its endogenous promoter, does not accumulate in the vacuolar lumen in a *pep4Δ* strain. Pep4p-dependent proteases reside in the vacuolar lumen, and upon deletion of *PEP4*, proteins degraded by this system will accumulate in the vacuolar lumen.

A kinetic relationship between endocytosis and exocytosis may be required to maintain the environment of this subdomain. Constant endocytosis is required to maintain a polarized localization of Snc1p towards the bud tip [7]. Our data indicates

that Snq2p also requires constant endocytosis to remain concentrated towards the mother cell plasma membrane. Although these proteins localize in opposite regions of the plasma membrane, perhaps it is through a kinetic relationship between endocytosis and exocytosis to create an environment at the plasma membrane where proteins have different affinities for a particular domain.

The functional significance for Snq2p to be excluded from the daughter cell plasma membrane for the majority of the cell cycle is unclear. Why would the cell want to exclude Snq2p from sites of exocytosis? Perhaps it is due to steric reasons. Snq2p is a large ATPase pump that regulates multidrug export. It may be excluded because the function is not solely dependent on spatial localization, as small molecules can be pumped out of the cell even when the pump is only localized to the mother cell. Therefore, space at the daughter cell plasma membrane might be preserved for the proteins that require spatial localization at this region for function. Our study suggests that yeast cells can have the ability to, not only concentrate proteins in the daughter cell plasma membrane, but also to organize proteins into a subdomain concentrated away from sites of exocytosis.

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Chapter 10. Conclusion

Intracellular transport is essential for eukaryotic cell growth. Rab GTPases serve as a focal point in the regulation of vesicular and organelle transport where they act as guanine nucleotide-dependent switches to regulate targeting to the acceptor compartment [1, 2]. Therefore, regulating the nucleotide state can drastically impact transport. *ELP1*, was identified as an extragenic suppressor of a guanine nucleotide exchange factor (GEF) that regulates exocytosis, *SEC2* [3]. The analysis in Chapter 4 characterizes a role for Elp1p and the Elongator complex, for which Elp1p is a subunit, and implied a role in negatively regulating exocytosis.

Phenotypic analysis of Elongator function impact on membrane transport

Additional studies extended this analysis to investigate how the Elongator complex regulates secretion. Data presented in Chapter 5 used exocytic mutants to characterize the effect of Elongator function on exocytosis and found that loss of Elongator function resulted in a similar suppression profile to overexpression of the Rab GTPase *SEC4,* the t-SNARE *SEC9* and to a lesser extent a Sec4p effector protein that regulates SNARE function *SRO7*. Another analysis discussed in Chapter 5, identified the SNARE modifier *SWF1* as a gene that phenocopies multiple loss-of-Elongator phenotypes [4, 5]. Therefore, two independent analyses show common physiologic effects on growth between Elongator function and SNARE function. Our current model places Elongator function downstream of Sec4p as potentially regulating the ability to interact with Sec9p and Sro7p.

Future studies to investigate interactions between exocyst subunits and Elongator-associated proteins

Analysis presented in Chapter 5 finds that three Elongator-associated proteins, *KTI11, KTI12* and *KTI13*, are required for the regulation of exocytosis [6-8]. Interestingly, two studies in human cells have found that knockdown of the human homologues of *KTI11* (DelGIP) and *KTI13* (DelGEF) increase secretion of a specific proteoglycan in humans [9, 10]. Furthermore, DelGEF directly interacts with the exocyst subunit Sec5 in a Mg^{++}-dependent manner and DelGIP enhances the interaction. The authors speculate DelGEF and DelGIP might bind Sec5 and regulate exocyst assembly in response to specific signaling cues. This is particularly interesting in the context of our analysis as the human homologues of two Elongator-associated proteins negatively regulate an aspect of secretion.

I have generated functional tagged version of Kti11p, Kti13p and Sec5p for expression in yeast that will be useful in the future to determine if this interaction also occurs in our system (Figure 10.1). Sec5p-MBP functionality was assessed through complementation of a *sec5-24* temperature sensitive mutant. Tagging Sec5p at the N-terminus with MBP results in a partially functional protein and therefore the tagged version at the C-terminus should be used for future studies (data not shown). Deletion of *KTI13* causes caffeine sensitivity (Chapter 5) and this phenotype was utilized to assess functionality of *myc* tagged constructs. *KTI11* was tagged with an HA epitope at the C-terminus and was cloned into single copy and multicopy plasmids, providing a system to test if, similar to the human studies, Kti11p enhances an interaction between Kti13p and Sec5p. If this interaction does occur in yeast, it will be important to determine if the interaction is Elp1p-dependent, as this will provide important insights to the role of the Elongator complex in exocytosis. Furthermore, using various exocyst mutants or exocyst subunit antibodies, the hypothesis that the

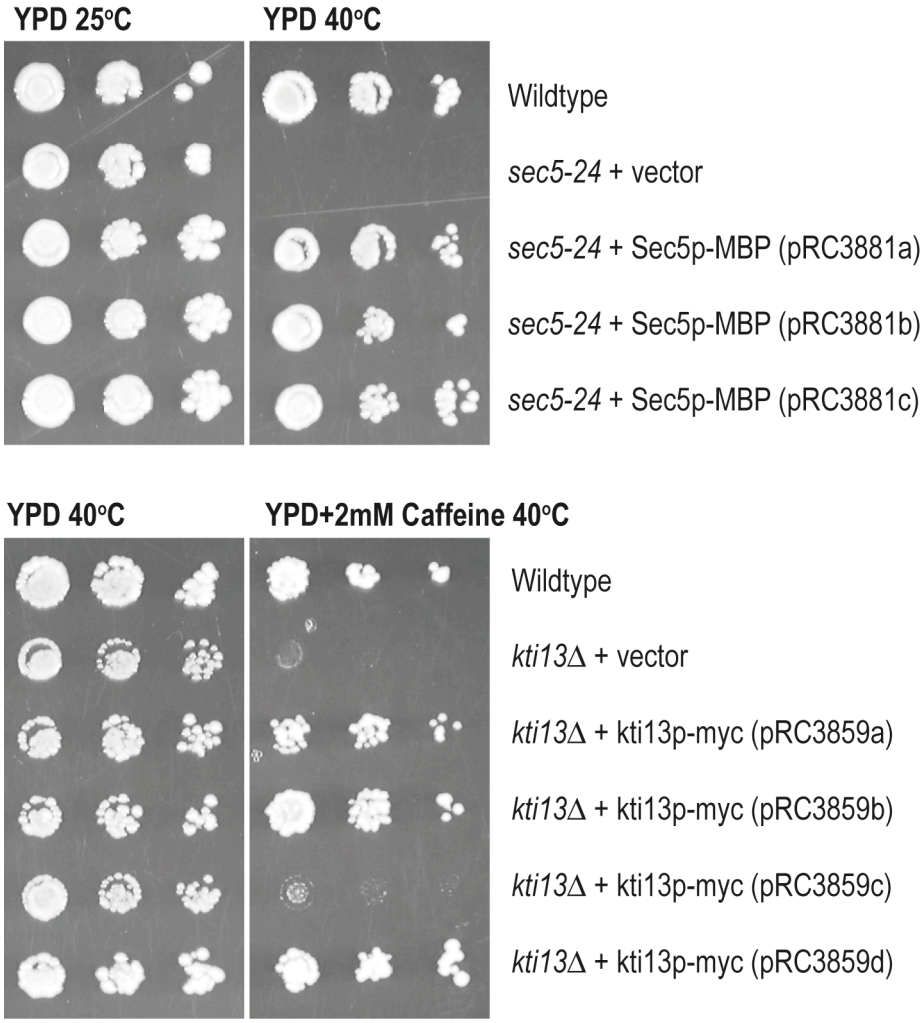

Figure 10.1. Functionality tests of tagged Kti13p and Sec5p. *top*. Three independently generated MBP-tagged Sec5p constructs (at the C-terminus) were transformed into *sec5-24* mutant cells along with a vector only control. Transformants were tested for growth on YPD 25°C (permissive) and YPD 40°C (restrictive). Functionality can be assessed by complementation for growth at restrictive temperature. *bottom*. Four independently generated myc-tagged Kti13p constructs, on single copy plasmids, were transformed into *kti13Δ/kti13Δ* cells along with a vector only control. Transformants were tested for growth on YPD 40°C and YPD+2mM caffeine 40°C. Functionality can be assessed for by growth on YPD+2mM caffeine 40°C.

interaction may regulate exocyst assembly could be addressed. It will be interesting to determine if this interaction occurs in our system, as it will provide insights into the mechanism for Elongator action in exocytosis and provide evidence for evolutionary conservation of Elongator-dependent exocytosis regulation.

Role of urmylation in regulating exocytosis and potential role in cargo-specific sorting

Data presented in Chapter 7 found that the ubiquitin-like urmylation pathway plays a role in the regulation of exocytosis. The regulation is conjugation-dependent as the E1 enzyme is required for the negative regulation (Figure 7.3). Little is known about the cellular process(es) regulated by urmylation, although it has been shown to be involved in regulating budding and intracellular amino acid levels [11-13]. This is consistent with a role in the TOR signaling pathway that is involved in nutrient sensing and regulating cell proliferation [11, 12, 14]. Loss of Elongator or urmylation function suppresses the temperature sensitivity of *SEC2* conditional mutants, however, it is still unclear if these two functions are in the same pathway or in parallel pathways (Figure 7.9), although other reports have linked these two processes [12, 15].

To further characterize the urmylation pathway, we have begun biochemical analysis to identify Urm1-conjugated proteins. The identification of urmylation substrates will provide key insights into how urmylation, and potentially Elongator, regulate exocytosis. Additional genetic analysis could be used to identify the membrane transport-relevant targets of urmylation to further understand the mechanism of regulation.

Perhaps Elongator and urmylation are components of a pathway to regulate exocytosis in order to control intracellular amino acid or nutrient levels. Through negatively regulating exocytosis, this pathway may regulate the presence of amino

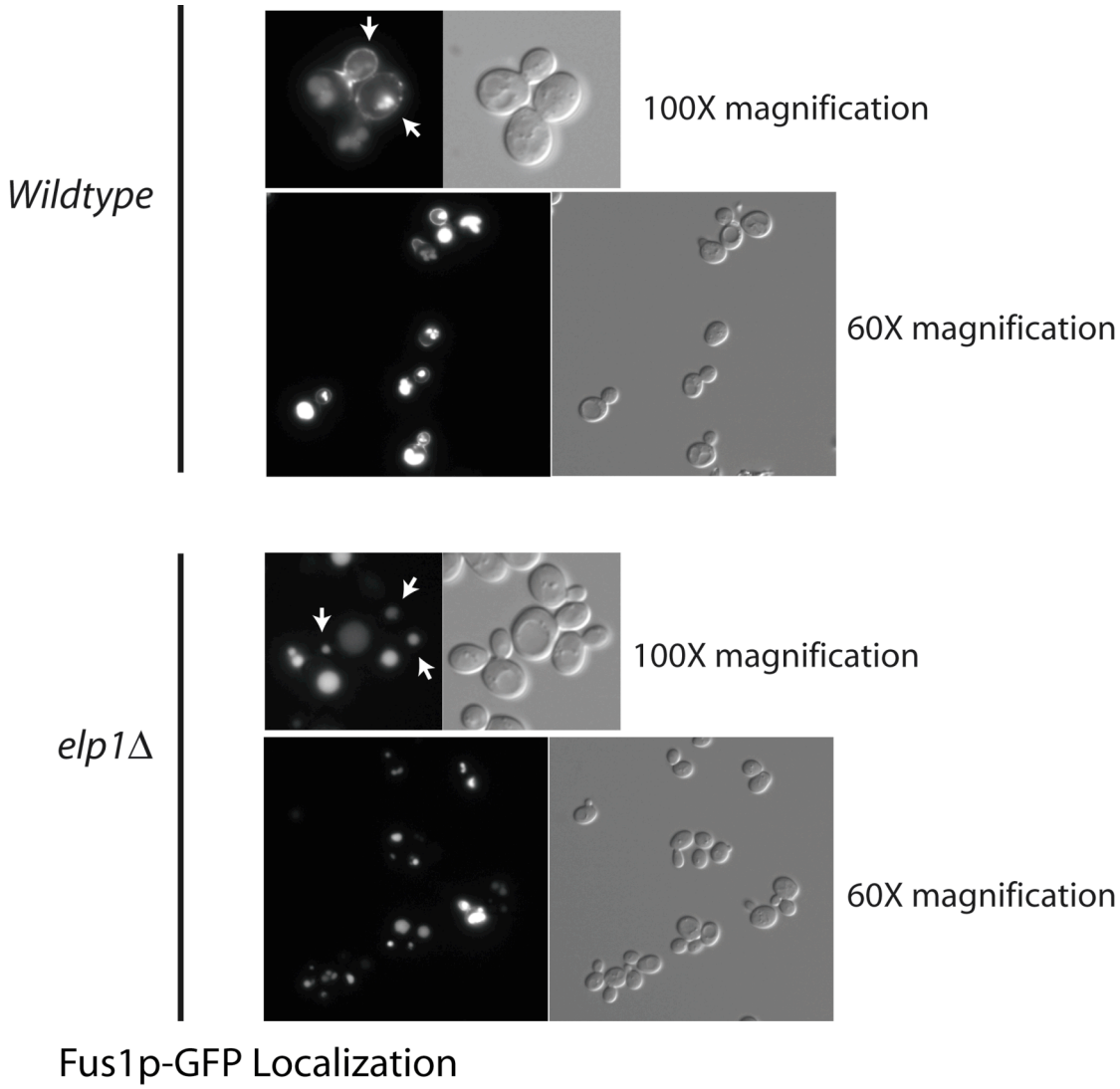

Figure 10.2. Fus1p-GFP localization in wildtype and *elp1*Δ cells. Isogenic wildtype and *elp1Δ* cells were transformed with Fus1p-GFP P_{GALS}. Transformants were grown in sucrose containing media for approximately 24 hours. These cultures were used to inoculate a fresh sucrose containing culture and grown overnight at room temperature. The cultures were transferred to galactose-containing media (2%) for six hours and grown at room temperature to induce Fus1p-GFP protein synthesis. Fus1p GFP localization was analyzed after six hours of expression using a 60X lens to collect images from a large field of view, and using a 100X lens to collect images with high magnification. Equivalent exposures were used for fluorescent images. Arrows indicate area of daughter cell plasma membrane.

acid transporters, such as Gap1p, at the cell surface. The GSE complex is a putative endosomal coat complex that regulates Gap1p transport to the plasma membrane [16]. Two GTPase subunits of this complex, Gtr1p and Gtr2p, were identified in the caffeine sensitivity screen presented in Chapter 5. Furthermore, deletion of *GTR1* suppresses the temperature sensitivity of *sec15-1* mutants, similar to deletion of Elongator (Figure 5.14). Perhaps Elongator and urmylation regulate transport of specific cargo to the plasma membrane.

We have found preliminary evidence for this through extending the analysis from a previously reported study. The *S.cerevisiae* genome was screened to identify genes required for proper transport of a Fus1p-Mid2p chimera protein to the cell surface and found that *ELP3* is required for this process [17]. This led us to analyze each full-length protein in cells lacking Elongator function. Fus1p-GFP, a protein required at the cell surface for mating, and Mid2p-GFP, a cell wall sensor, localization was analyzed in *elpΔ* and isogenic wildtype cells. Figure 10.2 shows that in wildtype cells, Fus1p-GFP localizes to the daughter cell plasma membrane and the vacuolar lumen. In *elp1Δ* cells Fus1p-GFP is predominantly localized to the vacuole and excluded from the daughter cell plasma membrane, suggesting altered trafficking in cells that have lost Elongator function. Mid2p-GFP localization was unaffected by deletion of *elp1Δ* (data not shown). These same observations were made for *elp2-elp6Δ*, suggesting a cargo-specific role for Elongator. Future studies could focus on studying the trafficking patterns on Gap1p and other amino acid transporters in *elp* and *urm1* delete cells.

Models for regulation of exocytosis through Elongator function

The exact mechanism for Elongator action in the regulation of exocytosis is unclear. In addition to a role in membrane transport, Elongator has been shown to

influence translation and transcription [18-20]. Analysis described in Chapter 4 demonstrates the regulation of membrane transport occurs independently of transcriptional regulation. We found that functional GFP-tagged versions of each subunit localizes to the cytoplasm and do not shuttle between the nucleus and cytoplasm. Furthermore, restricting Elp1p-GFP to the nucleus through attaching a nuclear localization sequence results in a nonfunctional proteins, indicating Elp1p has an essential role in the cytoplasm. A recent report suggests that Elongator's role in translation, through its requirement for the generation of modified uridine at the wobble position, is the direct function of Elongator and it regulates exocytosis as a secondary effect [21]. The lines of evidence for this come from experiments where the authors found overexpression of two tRNA species, *tQ(UUG)L tK(UUU)L* (*tQ, tK*), bypass Elongator's exocytic function as it restores the thermosensitivity of a *sec2-59 elp1Δ* strain, as well as restore Sec2p-GFP polarized localization in an *elp1Δ* strain. These two tRNA species were initially identified as they could suppress the caffeine sensitivity of loss of Elongator function.

Connections between translation and exocytosis

Analysis discussed in Chapter 5 verifies that overexpression of the tRNA species suppresses the caffeine sensitivity caused from deletion of each Elongator subunit (Figure 5.9a, Figure 5.15). Using the constructs generated for this test, *sec2-59 elp1Δ* thermosensitivity was not restored through overexpression of *tQ tK* (Figure 5.9b). This is an area that needs to be addressed further, most likely through obtaining the construct generated by the Bystrom laboratory in their initial studies [21]. When analyzing growth on YPD+2mM caffeine 40^{o}C in *elp1Δ* cells with the addition of *ELP1* or *tQ, tK*, it appears that overexpression of the tRNAs does not restore growth as

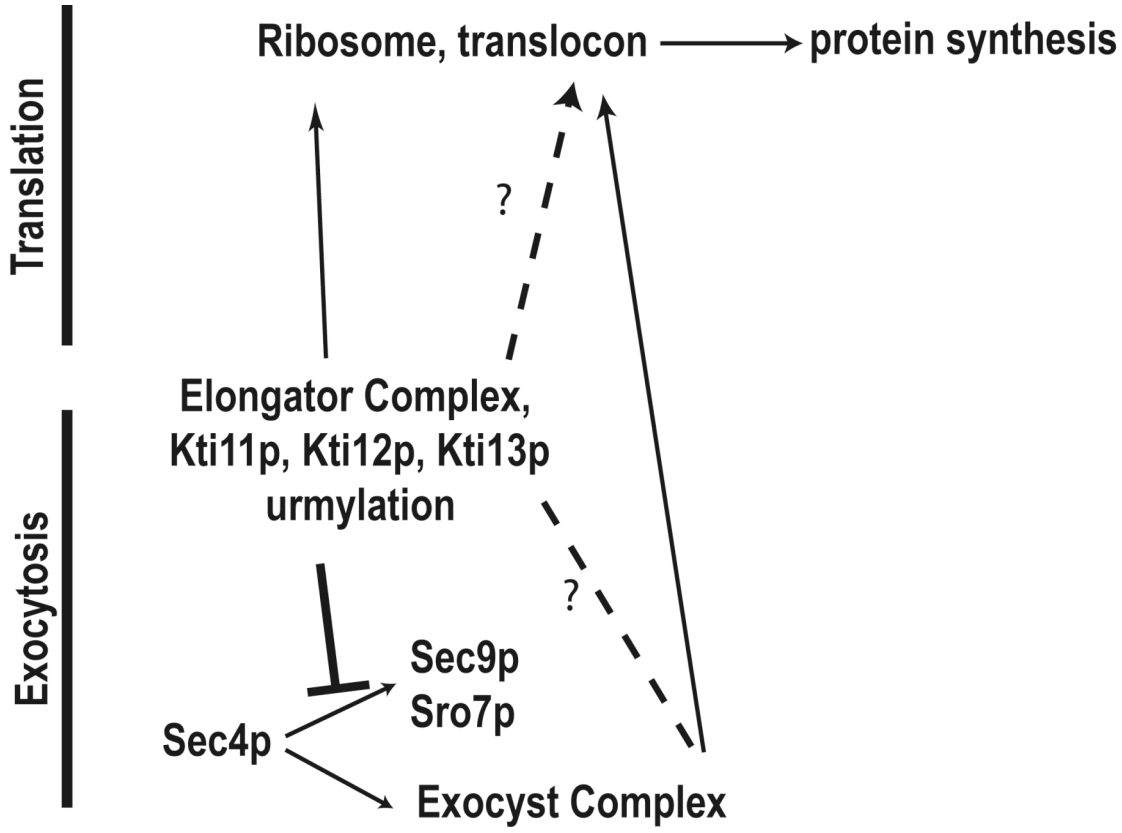

Figure 10.3. Model for Elongator and urmylation action to negatively regulate exocytosis.

well as providing a copy of *ELP1* (Figure 5.15 *top*). This may be due to tRNA expression levels or suggest additional functions for Elongator.

Cells lacking Elongator or Elongator-associated protein function, *KTI11, KTI12* and *KTI13*, do not have modified uridine at the wobble position [18]. Because of the link to translation, it is surprising that roughly 40% of the genes identified in the caffeine sensitivity screen are genes that encode proteins with known roles in membrane transport. *TOR1* was the only protein involved in translational events identified, with the next closest gene *SPC2*, a subunit of the signal peptidase complex that cleaves the signal peptide of proteins that have passed through the translocon [22, 23]. The exact role for Elongator in the biosynthetic pathway to generate modified

uridine is unknown. Furthermore, the mechanism for how overxpression of *tQ, tK* lead to the bypass phenotypes is unclear.

Phenotypic data suggests a physiologic link between the *tQ, tK* species, Swf1-dependent palmitoylation, and Elongator function (Chapter 5). Reports in the literature have identified physiologically relevant interactions between subunits of the exocyst complex and the Sec61 subunit of the translocon, therefore, providing precedence for a link between translation and exocytosis [24-27]. In mammalian cells, overexpression of the exocyst subunit hSec10 causes an overall increase in protein production of secreted proteins despite constant mRNA levels, essentially providing a feedback loop between exocytosis and protein synthesis [25, 27]. Thus, there appears to be signaling from the secretory pathway to the protein synthesis machinery that adjusts protein synthesis to the cell's secretory capacity.

The studies presented in this thesis have begun to characterize a pathway, or pathways, that negatively impact exocytosis. We have found that the Elongator complex, Elongator complex-associated proteins Kti11p, Kti12p and Kti13p, and the urmylation conjugation system have roles in negatively regulating exocytosis. Specifically, through genetic analysis, our current model places Elongator function at regulating excytosis upstream of the Rab Sec4p and downstream of Sec9p and Sro7p (Figure 10.3). The translational role for Elongator could imply that this complex serves as a component of the feedback loop that has been observed coupling secretion to protein synthesis [24-27]. Elongator may fine tune secretion rates in response to the translation capacity of the cells.

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